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A CRITICAL REVIEW OF HUMAN HAEMOGLOBIN VARIANTS

PART I: METHODS FOR SEPARATION AND CHARACTERIZATION

BY

G. H. BEAVEN AND W. B. GRATZER

From the Medical Research Council Laboratories, Hampstead, London, N.W.3

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At the time of writing, the existence of some 29 haemoglobins, both normal and pathological, seems to have received widespread acknowledgment. A number of excellent reviews (Allison, 1958; Betke, 1956, 1958; Itano, 1956; Itano, Bergren, and Sturgeon, 1956; Malassenet, 1957; Itano, 1957a; Huisman, 1958) have appeared in recent years describing the incidence, nature, and clinical and genetic manifestations of these haemoglobins, but making for the most part little mention of their characterization or of the available criteria for heterogeneity, on which the evidence for the existence of many of the haemoglobin variants rests. It is felt that a critical examination of these underlying factors together with an attempt to evaluate the results achieved by the various techniques which have been exploited is timely. In particular attention will be focused on a number of factors arising out of the inherent nature of haemoglobin which may be expected to have a fundamental bearing on the interpretation of experimental results.

Mention must first be made of the techniques that have been applied to the comparative study of human haemoglobin variants.

METHODS IN THE STUDY OF HUMAN HAEMOGLOBINS

Zone Electrophoresis

Paper.—This method has had the most widespread application on account of its cheapness and simplicity. It should be noted that the evidence for a considerable number of abnormal haemoglobins is based solely on the results of paper electrophoresis experiments. It is not proposed to discuss the method here, since reviews of general techniques are available

(Bergren, Sturgeon, and Itano, 1954; White, Beaven, and Ellis, 1956; Goldberg, 1957), but mention should be made of one or two of its limitations. In the first place the behaviour of a protein on paper depends on a number of incalculable factors, in particular electro-osmotic and adsorption effects, and it is not generally possible to obtain absolute mobility measurements by this means, though the measurement of relative mobilities by comparing three strips carrying the sample, an Hb-A (adult haemoglobin) control, and a mixture of the two, run simultaneously, has been proposed by Toporek (1956). Irreversible adsorption can be largely eliminated by the addition of a fast-running, non-reacting protein to the buffer mixture (Hoch and Barr, 1956). The method remains an essentially comparative one, though here again attempts have been made to incorporate mobility markers into the system (Goldberg, 1957) or to run markers by way of control (de Wael and Punt, 1957). Heating effects are of importance and can give rise to spurious heterogeneity, and Topalović-Avramov, Draškoci, Kapetanović, and Lero (1956) have observed that substantial changes in the rate of migration of one serum protein component relative to another, amounting in extreme cases to inversion of position, is brought about by varying the distance between the point of application of the sample and the anode. Reference must here be made to a cautionary article by Moinat and Tuller (1957), who suggest that paper electrophoresis experiments be carried out in the cold room. The present authors believe that the results of paper electrophoresis have in many cases been insufficiently scrutinized, and a good example exists of the pitfalls into which the method can lead, in which the observations of an allegedly new haemoglobin reported from one laboratory (Battle and Lewis, 1954) could not be repeated with the same sample under rigorous conditions (Itano *et al.*, 1956).

Typical results obtained by paper electrophoresis are given by Motulsky, Paul, and Durrum (1954), Chernoff (1955), Smith and Conley (1953), Larson and Ranney (1953), Reynaud (1953), and other workers, and a comparison of results in different buffers is given by Morrison, Rudnicki, and Diggs (1954). The separation of Hb-A and Hb-F (foetal haemoglobin) has been observed on paper only by Gatto and La Grutta (1955a) and by Haynie, Dobson, and Hettig (1957) in cases where Hb-F is present to the extent of 40% or more. A good estimate of lower Hb-F concentrations has been claimed by the former authors in terms of rate of migration of the compound zone compared with that of pure Hb-A.

Quantitative work by dye-elution or staining and subsequent photometry has been carried out (Larson and Ranney, 1953; Ranney, Larson, and McCormack, 1953; Modiano, 1957; Silvestroni and Bianco, 1957), though it is conceivable that the results are in some cases rendered ambiguous by the non-specificity of the stains which have been used (Modiano, 1957; de Traverse, Coquelet, and Brumpt, 1958). Other limitations on the accuracy of such work (Shooter, Skinner, and White, 1958) exist (*v. infra*).

Paper electrophoresis is ill adapted for preparative work, though attempts at elution have been made (White *et al.*, 1956). Scheinberg, Harris, and Spitzer (1954) have described a "differential titration" method on paper, which sets out to obtain information on the number of charged groups in different haemoglobin variants. The conclusions (see also Scheinberg in N.A.S.-N.R.C. Conference, 1958, p. 277) are, however, seen to rest on experiments carried out at extreme *p*H values, at which the haemoglobins are extensively denatured, and are accordingly of limited validity.

Recently good results have been obtained by the use of cellulose acetate in place of paper (Kohn, 1957).

Starch Gel.—This method was devised by Smithies (1955) and has been successfully applied to human haemoglobins by Owen and Got (1957), who have achieved the difficult Hb-A/Hb-F separation in a striking manner, and also by de Grouchy, Schapira, and Dreyfus (1958). The method has preparative possibilities since the haemoglobin can be extracted from the segregated zones by electrophoresis (Moretti, Boussier, and Jayle, 1958) or by freezing (in the latter case at the expense of some denaturation) (Smithies, 1955).

Starch Block (Kunkel, 1954).—The electrophoresis of haemoglobin in beds of starch grains was described by Kunkel and Wallenius (1955) and has been used by Kunkel and his co-workers for the isolation of the A_2 component of adult blood (Kunkel and Bearn, 1957; Kunkel, Ceppellini, Müller-Eberhard, and Wolf, 1957).* The method lends itself particularly well to preparative work since the pigment is easily extracted, and Kunkel and Bearn (1957) have run 20 ml. samples of 8% haemoglobin solutions on large starch blocks. The technique has been used for routine analysis by Bernasconi (1956), Marinone, Bernasconi, Morsiani, and Lucci (1956), Marinone and Bernasconi (1957), and by Gerald and Diamond (1958a), who applied their samples in the form of cyanmethaemoglobin, a derivative whose use is rendered particularly desirable in virtue of its relatively great stability. Recently Masri, Josephson, and Singer (1958), making use of electro-osmotic flow on starch block, have obtained three minor haemoglobins as well as some non-haemoglobin fractions from normal adult haemolysates (see also Ceppellini, Kunkel, and Dunn, 1958). A remarkable separation of haemoglobins F and A has very recently been reported by Künzer and Ambs (1958) by the use of very high voltages on starch slab. This may well be a major advance in the problem of haemoglobin fractionation.

Agar Gel.—Electrophoretic separation of haemoglobins in agar gel was described by Giri and Pillai

*Kunkel *et al.* (1957) have also used polyvinyl chloride as supporting medium in much the same way.

(1956), who were able to distinguish between haemoglobins A and S at *p*H 8.6. Giri, Rao, and Natarajan (1958) also demonstrated the presence of minor protein components in human red cell haemolysates by the same means. Robinson, Robson, Harrison, and Zuelzer (1957) devised a method of separating haemoglobins at low *p*H, and have obtained good results, notably in the resolution of Hb-F.

It should be mentioned that the order of migration of the haemoglobins on agar (Robinson *et al.*, 1957) differs in some instances from that obtaining in other media. It is to be surmised that migration of haemoglobins on agar involves adsorption or molecular-sieve effects. Disagreeable artifacts can occur, apparently by overloading (Robinson *et al.*, 1957). A method of electrophoresis in a liquid film containing a small quantity of agar has been described (Ressler, Nelson, and Oster, 1958), and has been applied to haemoglobins by Zak and co-workers (Zak and Jarkowski, 1958; Zak and Sun, 1958). A micro-electrophoresis method on agar has also been reported (Monnier and Fischer, 1957, 1958).

Continuous Electrophoresis.—A number of methods of continuous electrophoresis for preparative use have been devised (Brattsten, 1955), and one such has been applied successfully to the fractionation of turtle haemoglobins by Ramirez and Dessauer (1957b).

Free Boundary Electrophoresis

This technique has found wide application in the study of haemoglobin (Zinsser, 1952; Itano *et al.*, 1956; van der Schaaf and Huisman, 1955b, etc.) though the results have not been unequivocal. It has the particular merit of yielding mobility values for each species under given conditions, and from these the iso-electric points can be interpolated. Large samples are required, and though the method was used for the isolation of Hb-E (Itano *et al.*, 1954) its preparative use is small. A detailed theoretical discussion of the free boundary technique and its limitations, together with its specific application to the fractionation of Hb-A and Hb-F, is given by Hoch (1950), and Shooter and Skinner (1955) discuss the optimal conditions for resolution of Hb-S (sickle-cell haemoglobin). The drastic dependence of the results obtained for proteins on the selection of a suitable buffer is discussed by Woods (1958).

Micro-electrophoresis

The micro-electrophoresis technique of Antweiler has proved inadequate for the study of such closely related species as the human haemoglobin variants (Waris, 1954).

Iso-electric Line Spectra

The interesting iso-electric line spectra of Kolin (1955) have been obtained with human haemoglobins by Tuttle (1956). This is evidently a method of considerable potentialities, in virtue of its simplicity and its fundamental nature, since separation depends solely on the iso-electric points of the species present.

Chromatography

Paper.—The paper chromatography of haemoglobins is of questionable value (Vella and Lugg, 1957). In all work of this type solvents have been used which do violence to the haemoglobin molecule, and the results would seem to defy interpretation (Fiori, 1957; Sansone, 1952; Sansone and Cusmano, 1950; Sansone, Cusmano, and Ravazzoni, 1951; Andersch, 1953), though consistent diagnostic results have been claimed (Berlingozzi, Rapi, and Dettori, 1953; Rapi, Dettori, and Salvioli, 1956; Illari and Marenghi, 1955; Penati, Lovisetto, Turco, and Abrate, 1954a). Penati, Lovisetto, Turco, and Hutter (1954b) state that they can distinguish clearly between samples of haemoglobin from normal and thalassaemic subjects by the shape of the pigment smear in two-dimensional paper chromatography, using 50% pyridine as eluent in both directions. Goldberg (1958a) has investigated the electrophoresis of haemoglobins on paper impregnated with a micro-powder of "amberlite IRA-400," a strongly basic ion-exchange resin, and here again the results are questionable, two, and sometimes three, major components being obtained.

A method has also recently been described (Jain, 1958) for separating haemoglobin components on paper with a superimposed *p*H gradient. Successive precipitation is said to occur and three bands have been obtained from Hb-A. The significance of the separation is as yet in doubt.

Starch and Alumina Columns.—An exhaustive study of the behaviour of adult and foetal haemoglobins on starch columns has been made by Penati and co-workers (Penati, Turco, Hutter, and Lovisetto, 1955e), who conclude that no useful results are likely to accrue.

Alumina columns have given rise to debatable results in the hands of Kruh (1952) and of Van Fossan (1954) (*v. infra*). Prins and Huisman (1956a) found that the apparent heterogeneity of haemoglobin samples on alumina columns varied with experimental conditions, and discarded this technique.

Ion-exchange Columns.—Ion-exchange columns were first used for the fractionation of haemoglobins by Boardman and Partridge (1954, 1955), who separated mixtures of animal haemoglobins under carefully controlled conditions on the cation-exchange resin "amberlite IRC-50." (It may be mentioned that Boman (1955) has shown that elution of haemoglobin from an anion-exchange column under mild conditions is possible, but no use appears as yet to have been made of this observation.) No adequate separation of human Hb-A and Hb-F mixtures has been attained under the conditions of Boardman and Partridge (Beaven and Gratzer, 1958). Their procedure was modified by Morrison and Cook (1955b, 1957), Cook and Morrison (1956), and Allen,

Schroeder, and Balog (1958) and used to resolve minor components in normal adult blood, and also by Huisman and co-workers (Huisman and Prins, 1955, 1957; Prins and Huisman, 1955). The latter workers have developed two techniques: first, elution of haemoglobins from columns involving step-wise changes of buffer, a procedure which invites criticism on the grounds that spurious peaks can be generated by the assimilation of the tail of a zone by the new buffer, and secondly, the development of zones in a flat cuvette under conditions where the R_f value of the protein is too low to permit of elution at a useful concentration. It may be pointed out that the procedures described by Huisman and Prins (1955) imply incomplete equilibration of the resin with the buffer, and also depend on non-equilibration of the protein sample, since dialysis against the buffer to be used is specifically avoided. Whilst not calling into question the diagnostic value of Huisman's cuvettes, the authors have found that fractionation of Hb-F and Hb-A under these conditions is partly illusory. Mixtures of equal parts of Hb-F and Hb-A in the carboxy form were applied to columns under the condition specified by Huisman and Prins, and examination of the emergent zones spectrographically showed that separation of the two proteins was incomplete. The same was found to be true under cuvette conditions, when the separate pigment zones were extracted and analysed.

Huisman (1957b) gives figures for the percentage of Hb-F in mixtures applied to columns and the percentages of Hb-F and Hb-A in the zones ascribed to these components. These reveal that in general, and especially where the proportion of Hb-F is large, the zone identified with Hb-F contains up to some 16% more haemoglobin than the total Hb-F applied in the mixture. If Huisman's photometric methods of estimation are correct, imperfect fractionation is clearly indicated. This may be identifiable with similar effects observed in electrophoresis (Shooter *et al.*, 1958) (*v. infra*).

It may be anticipated (*v. infra*) that the separation of Hb-A and Hb-F may cause more difficulty than that of most other pairs of human haemoglobin variants, but the need for a greater degree of scepticism in the interpretation of chromatographic phenomena is indicated. The work of Morrison and Cook (1955b) on the separation of minor components from normal adult haemoglobin is also evidently not unambiguous.

Allen *et al.* (1958), using phosphate buffers differing slightly in composition, obtain three minor components from Hb-A and at least one from Hb-F. These components are obtained by separation first with one buffer, followed by fractionation of one of the zones with another buffer. The results are not consistent with those of Morrison and Cook (1955b, 1957), both in the absence of any slow-running component whatever and in the proportions of minor components involved, but can be reconciled in part with the appearance of small, fast-running components from Hb-A and Hb-F on the columns of Prins and Huisman (1956b). Allen *et al.* suggest that the difference in *iso*leucine content between the various zones, as revealed by analysis, is an indication of the validity of their results. On the other hand, since it has been shown that variations in *iso*leucine content can arise from impurities (Stein, Kunkel, Cole, Spackman, and Moore, 1957), the possibility must be borne in mind that the fractionation may arise from binding of haemoglobin with extraneous substances with or without a large *iso*leucine content. The alleged substantial difference between the *iso*leucine contents of oxy- and of carboxyhaemoglobin A (Derrien and Laurent, 1955a; Allen *et al.*, 1958), whether crystallized or not, also renders such an explanation possible.

The authors have examined the behaviour of haemoglobins on modified cellulose columns. These materials developed by Peterson and Sober (1956) have certain advantages over resin, in particular their large capacity. Partial fractionation of Hb-F/Hb-A mixtures has been achieved, but a number of remarkable artefacts have also been observed, where two well-defined zones appeared immediately on application of buffer and were found to have identical compositions. Huisman has reported successful fractionation on columns of modified cellulose (Huisman, 1957a; Huisman, Martis, and Dozy, 1958).

Solubility

The solubility of a haemoglobin, both in the oxy- or carboxy as well as in the reduced form, constitutes a valuable parameter for its characterization and at times identification (a notable example is the use of this method for distinguishing Hb-D from Hb-S). Moreover, the appearance of the solubility curve of a protein has assumed classical status as a criterion for heterogeneity. The application of this method to the human haemoglobins is discussed in detail by Itano (1953a, 1957a). On the other hand, it seems clear that unwarranted conclusions have often been drawn from the results owing to a failure to appreciate the limits of the accuracy of which such work is capable.

Two procedures are available, the variable solute and variable solvent (salting-out) methods. It must first be said that many haemoglobin mixtures give rise to solid solutions (Itano, 1953a, 1957a), and in such a case the concentration of protein in solution when plotted against the total concentration in the solid phase will give a smooth curve. The smaller the number of readings available the more likely is it that a false impression of heterogeneity will be gained. (If in fact all successive points are joined by straight lines, the plot will indicate the presence of as many components as there are points.) In the absence of solid solutions the detection of discontinuities in the solubility curve is still somewhat subjective. Goldberg (1958a) has carried out an

analysis of experimental precision in the measurement of the solubility of reduced haemoglobin in a phosphate buffer under fixed conditions. Samples tested with 10 preparations of the same buffer gave results varying within limits of 2% and duplicate determinations on 35 samples of Hb-A gave solubility variations of up to 14%, with a standard deviation of 3.22. (It was also noted that lower solubilities were obtained with samples which had been kept at ordinary temperatures for any length of time.) An examination of most published work on solubility curves, most of all that of Derrien and co-workers (*v. infra*), shows that the evidence for the presence of minor components in the protein is frequently based on deviations from the curve lying well outside these accuracy limits.

An example of the application of the variable solute technique to normal adult haemoglobin is given by Allison and Tombs (1957). These authors find that three components are present in the system, but Itano (1953a) is of the opinion that the method is insufficiently sensitive to detect the minor components revealed by electrophoresis (Kunkel and Wallenius, 1955; Kunkel *et al.*, 1957; etc.) and ion-exchange chromatography (Morrison and Cook, 1955b, 1957; Allen *et al.*, 1958) and present to the extent of only a few per cent. He finds that, within the limits of his own experimental accuracy, normal adult haemoglobin is shown to be homogeneous.

The same strictures must be said to apply to the salting-out (variable solvent) procedure. Certainly the minor components of normal adult blood, as demonstrated by other methods, could scarcely explain the proliferation of haemoglobins in the systems examined by Roche, Derrien, and co-workers (Roche and Derrien, 1951; Roche, Derrien, and Roques, 1952b, 1953a; Roche, Derrien, Gallais, and Roques, 1952a; Roche, Derrien, Diacono, and Roques, 1953b; Roche, Derrien, Reynaud, Laurent, and Roques, 1954). A number of explanations for the appearance of misleading discontinuities suggest themselves. Itano (1956) believes that changes in aggregation of the haemoglobin molecules due to interaction with smaller molecules or with ions may be responsible. Evidence of such changes under the influence of ions has been adduced on the basis of light-scattering experiments, but results of different workers conflict (Benhamou and Weill, 1956, 1957; Putzseys, Schonne, and Sempoux, 1955). More significant are the observations of Karvonen and Leppänen (1952a) working with sheep haemoglobins. These authors established a clear-cut correlation between the sodium ion concentration within the parent erythrocyte and the crystal habit of the haemoglobin. It was found that where the sodium ion concentration was low, precipitation of needles occurred at *p*H 7, whereas in other samples precipitates of plates were formed at *p*H values below 6. The solubility of the samples was found also to increase with the total haemoglobin concentration. Such effects, as Karvonen and Lepännen point out, could arise not only with a protein mixture but also from the presence of intra-erythrocytic impurities in the solution. In a further study (1952b) these authors observed that the tendency of the haemoglobin to remain in a supersaturated form, when not even the appearance of the first crystals brought about an immediate "autocatalytic" precipitation, was substantially reduced if it had previously been precipitated in amorphous form. This is ascribed to the presence of impurities, which act as protective colloids, and remain in the mother liquor after precipitation. Such manifestations are clearly not to be ignored.

Another suggestion which has been made (Itano, 1953a, 1957a) to explain the superabundance of reported discontinuities in the salting-out curves is the occurrence of phase transitions at the high salt concentrations at which the experiments have to be carried out. A phenomenon of this kind was observed by Ogston and Tombs (1956) to occur in β-lactoglobulin, and they have proposed a simple criterion for ascertaining whether breaks in the solubility curve arise from true heterogeneity or from a phase transition. In the former case, the position of the break in the curve will depend on the initial protein concentration, whereas in the latter this will have no effect. It follows also that if the break is indeed due to a phase transition a minimal initial protein concentration must exist which will give a curve without breaks. This behaviour has not yet been reported for haemoglobins, but the A, F, and S variants are known to crystallize in more than one system (Perutz, Liquori, and Eirich, 1951; Jope and O'Brien, 1949). The onset of such phase transitions under experimental conditions must be regarded as a possibility; on the other hand it seems by no means unlikely that a given crystal form will persist in metastable state over a considerable range of conditions.

The authors believe, however, that a thorough scrutiny of the experimental parameters underlying the variable solute results of Derrien and co-workers is particularly desirable before any conclusions are drawn from them. It is hard to believe that experimental error might not absorb a number of the subtler deviations observed in the salting-out curve, which are greatly magnified in the differential graphs which are drawn on the basis of the changes in slope. (It may be noted that Polosa, Motta, and Lo Turco (1958) find that the magnitudes of the fractions obtained in this way from the haemoglobins of various animals vary with the individual.) In the first place, it is quite clear that in many cases the deviations of the points which define a discontinuity are not consistent with the limits of precision of solubility measurements set out by Goldberg (1958a) (*v. supra*). Secondly, the breaks, in the flatter parts of the curve particularly, depend on extinction measurements of accuracy lying very close to the limits for the standard type of spectrophotometer. Moreover, these depend again on the exact adherence to Beer's law of the dilution of aliquots over a wide range. The latter difficulties are circumvented by Pagliardi, Vitelli, and Gaidano (1954), who measure

protein nitrogen content rather than spectrophotometric extinction and obtain rather more convincing results, only small minor components of relatively low solubility, possibly representing impurities, being obtained from normal adult, cord blood, and thalassaemia haemoglobins. The reproducibility of the results of Derrien and co-workers may be explained at least in part by the fact that solute change increments are taken at the same points in all their experiments. Where a single point defines a discontinuity a peak or at least an inflexion appears on the differential plot.

It is clear that before any conclusions on the heterogeneity of haemoglobin solutions can be drawn from solubility curves a statistical analysis of the limits of experimental accuracy and precision is required, as well as a more detailed physicochemical examination of the system, both in respect of the solution and of the solid phase.

Chemical Behaviour

Alkali Denaturation.—The alkali denaturation of haemoglobins has been the subject of detailed study. For diagnostic purposes, that is to say, for the estimation of Hb-F in samples, a number of efficient routine procedures are available (Ponder and Levine, 1949; Singer, Chernoff, and Singer, 1951; Beaven and White, 1953; Derrien and Laurent, 1954; Chernoff, 1955).

In general, alkali denaturation experiments must be carried out with oxyhaemoglobin. Repeated attempts have been made to carry out measurements of the alkali denaturation rates of the carboxy and cyanmet derivatives, and Künzer (1953a, 1957a), using the precipitation method, has claimed that the results of such experiments are satisfactory for the estimation of Hb-F, and, indeed, that the use of cyanmethaemoglobin is preferable to that of oxyhaemoglobin. His success has not, however, been confirmed in other laboratories (Derrien, Laurent, and Roche, 1953; Beaven, Ellis, and White, 1958; Polosa, Motta, and Falsaperla, 1957). Künzer has suggested elsewhere (Künzer, 1955) that inconsistencies in results obtained with cyanmethaemoglobin can arise from contamination with and binding of plasma proteins. It seems likely that carboxyhaemoglobin must be converted to the oxy form before denaturation can proceed, and that the variations in denaturation rates from sample to sample depend on the amounts of excess carbon monoxide present (Introna, 1952; Derrien and Laurent, 1955b). Furthermore, Kubowitz in a comprehensive paper (1957) has shown that an intermediate of the denaturation of carboxyhaemoglobin is a carboxy pigment. Complications might also arise from the stepwise degradation of the carboxy derivative. Itano has shown that the four haems combine successively with carbon monoxide, and that three intermediates between haemoglobin and carboxyhaemoglobin can be isolated electrophoretically (Itano and Robinson, 1957).

The mechanism of the reaction remains obscure, though Kubowitz has shown that denaturation occurs by a number of contiguous or simultaneous processes, and that it is not justifiable to assume a true first-order mechanism, a fact which has also been stressed by Künzer (1953b) and by Rossi-Fanelli, Azzone, and Mondovi (1955a), who found that the values of the apparent first-order constants, and the proportions of the components deduced from them varied substantially with the *p*H. If such an assumption is in fact made, it could follow that changes in slope in the denaturation rate curve are due to heterogeneity of the sample, the overall reaction rate being compounded of the first-order rates associated with each component. This error has led to claims for the existence of three foetal and varying numbers of adult haemoglobins (Jonxis, 1949; White, Delory, and Israels, 1950; Betke, 1951; Kleinknecht, 1953). Betke (1952a, 1954) has shown neatly and conclusively that normal adult haemoglobin is not divisible into fractions of varying lability to alkali by dialysing the filtrate in a precipitation-denaturation experiment and showing it to be identical in its behaviour towards alkali with the original sample brought to the same concentration.

That different results are obtained by the two methods commonly used for following alkali denaturations, viz., the optical and precipitation methods, is well known (e.g. Jonxis and Huisman, 1956) and was indeed to be anticipated. Itano has shown furthermore (1957b) that, if the reaction is followed spectrophotometrically at different wavelengths, different rate curves are obtained. Each of these may correspond, though not uniquely, to concentration changes of the oxyhaemoglobin, alkaline haematin, or any intermediate products. The interpretation of such data in terms of the fate of the native protein is problematical. The precipitation method is equally arbitrary in its choice of a criterion for denaturation, and has been criticized on other grounds (Itano, 1957b), in particular that the precipitate might carry down with it some undenatured haemoglobin. This has not been the experience of Betke (1953a), who has shown that no haemoglobin is released from the precipitate by dilute ammonia. Betke also gives an interesting comparison between the reaction rates of oxy- and carboxyhaemoglobin as indicated by the two methods. Penati, Turco, and Lovisetto (1954c) give an equation relating the results obtained by the two methods.

Kubowitz (1954) has suggested that loss of oxygen or carbon monoxide affinity is a more satisfactory criterion of denaturation, and it does indeed seem particularly suitable for the study of haemo-proteins (*v.* also Kubo, 1956). Moreover, as a means of following alkali denaturation reactions, his manometric method is less ambiguous than the usual procedures. The rate of reaction as observed by this means is greater than that derived from optical measurements at the usual wavelengths. Kubowitz regards the values from the optical method as incorrect. One fundamental point appears, however, to

have been neglected, namely the possibility discussed below that methaemoglobin is formed in the first stage of the denaturation process. This would naturally lead to a loss in oxygen-binding capacity, even though the mere formation of methaemoglobin could scarcely be said to be consistent with any of the accepted definitions of denaturation. Kubowitz has in addition carried out measurements on reduced haemoglobin where the rate of formation of the end product (haemochromogen), as followed spectrophotometrically, agrees with the gasometric measurements, and has studied crystallized Hb-F and Hb-A, which were found to behave in the same way as the corresponding red cell haemolysates. Kubowitz has also been at some pains to show that the quantitative determination of Hb-F in admixture with Hb-A is to be relied on only when the proportion of Hb-F is substantial. This is borne out by the observations of Beaven *et al.* (1956), and observations of the presence of small alkali-resistant components in normal adult blood (Singer *et al.*, 1951; Huisman, Jonxis, and Dozy, 1955; Künzer, 1955) must be judged accordingly. It is interesting to note in passing that the isolated globin of Hb-F is abnormally prone to denaturation (Havinga, 1953).

Besides Hb-F two other characterizations of haemoglobins in terms of alkali denaturation rates have been claimed. The first is the pre-foetal haemoglobin (Hb-P) of Allison (1955). A haemoglobin was found by Halbrecht and Klibanski (1956) and Halbrecht, Klibanski, Brzoza, and Lahav (1958) in young foetuses which they claimed to be highly alkali-resistant. On the other hand, Künzer (1957b), Künzer and Drescher (1956), and Drescher and Künzer (1953, 1954) describe their pre-foetal component found under the same circumstances to be intermediate between Hb-F and Hb-A in its resistance to alkali. Recently a new "fast" haemoglobin, described as "Bart's" was reported by Ager and Lehmann (1958) in the presence of Hb-F in an infant blood and was stated to show resistance to alkali greater than Hb-A in the Singer one-minute test.

Acid Denaturation.—Attempts have been made to characterize Hb-F by acid denaturation but have produced little more than the fact that the resistance of Hb-F to acids is greater than that of Hb-A.

The rate of change of oxyhaemoglobin-A into acid haematin has been studied by Bugyi (1956), and Kleihauer (1957) has demonstrated by a precipitation method that Hb-F is two and a half to three times more resistant to hydrochloric acid than Hb-A. Putignano and Cognetti (1952) claim that carboxyhaemoglobin from the blood of thalassaemia patients is much more resistant to acids than the normal adult pigment. A more exhaustive study by Penati, Lovisetto, and Turco (1955c) has shown that the precipitation method cannot profitably be applied to acid denaturation since the differences in the rate curves for Hb-F and Hb-A are too small to give reliable extrapolated concentrations. Differences between the acid denaturation curves can be obtained by following the reaction spectrophotometrically, but are regarded as insufficient to permit of any useful conclusions.

Heat Denaturation.—This procedure has been applied by Betke and by Betke and Greinacher (1954) to the characterization of Hb-F (Betke, 1953c; Betke and Greinacher, 1954b) and Hb-S (Betke and Greinacher, 1955). The reaction is first order and indicative of homogeneity in Hb-A, Hb-F, and Hb-S. Mixtures of haemoglobin F and A give a denaturation curve characteristic of heterogeneity which can be expressed as the sum of the individual reactions. Hb-F is more susceptible to heat denaturation than Hb-A, differing by a factor of two to two and a half at 72° C. Similar behaviour is exhibited by the cyanmet derivatives. Hb-S shows no difference in its behaviour from Hb-A.

Other Denaturing Agencies.—The denaturation of haemoglobin with urea and with sodium salicylate has been studied in a number of connexions (*v. infra*).

A comparative study of methaemoglobins F and A was made by Gardikas, Scott, and Wilkinson (1953), who found that Hb-F was more resistant to sodium salicylate, but less so to urea.

Oxygen Dissociation.—The oxygen dissociation curves of the haemoglobins have been the subject of intensive study in virtue of their physiological interest.

Since the oxygen-binding capacity of haemoglobin is essentially a function of the haem iron, it might be considered surprising to find differences in affinity arising from aberrations in the globin portion. On the other hand it is now known (Riggs, 1952) that the haem–haem interactions which appear largely to govern the shape of the oxygen dissociation curve (see for example Wyman, 1948; St. George and Pauling, 1951) are strongly dependent on the sulphydryl groups in the molecule, and these in turn will be affected by any fairly drastic treatment of the pigment. It should be possible to explain in these terms the early observations of Geiger (1931), who separated animal and human haemoglobins cataphoretically into two components, present in bulk, which exhibited markedly different oxygen equilibrium curves. Moreover, the presence of unknown erythrocyte constituents (Allen, Wyman, and Smith, 1953; Becklake, Griffiths, McGregor, Goldman, and Schreve, 1955), as well as *p*H and ionic strength (Takashima, 1955, etc.), have their effects on the oxygen dissociation equilibrium, and consequently specific differences between, for instance, haemoglobins F and A in this respect (e.g., McCarthy, 1943) cannot at the present state of knowledge be accepted without reserve. Indeed, Allen *et al.* (1953) have shown that whereas fresh solutions of Hb-F gave values of oxygen

affinity consistently less by about 13% at the half-saturation point than Hb-A after simultaneous dialysis of the haemolysates from foetal and maternal blood against a common volume of buffer the oxygen affinity of the two haemoglobins became equal, the Hill constant remaining unchanged.

Oxidation and Reduction Reactions.—Künzer and Künzer (1952) observed that spontaneous oxidation to methaemoglobin occurred in cord blood more rapidly than in adult or infant blood. This has also been observed by Betke, Kleihauer, and Lipps (1956d).

Detailed kinetic studies of the oxidation of adult, foetal, and other haemoglobins have been carried out by Betke and, in the case of oxidation by molecular oxygen, a reaction evidently of some complexity, by Kiese and Schneider (1955). It was noted by Betke (1952b, 1953b) and by Künzer and co-workers (Künzer, Ambs, and Schneider, 1953; Künzer and Saffer, 1954) that foetal haemoglobin was oxidized more rapidly to methaemoglobin than the adult pigment. Künzer and Saffer give the ratio of half-reaction times with potassium chlorate as 1.59:1, and large differences with sodium nitrite are reported (Künzer *et al.*, 1953). Betke (1952b) and Betke, Greinacher, and Hecker (1956) followed the oxidation of reduced adult and foetal haemoglobins by potassium ferricyanide under various conditions of *p*H and at different initial concentrations of haemoglobin and methaemoglobin by an optical method, and found that under their conditions there were no perceptible differences between the behaviour of the two haemoglobins. Betke (1953b) also followed the reduction of the methaemoglobins by sodium dithionite by a streaming method, and showed that within the accuracy of his apparatus, which he gives as 5%, the behaviour of Hb-A and Hb-F was identical. The reduction of the oxyhaemoglobins was observed in the same apparatus, and, if the first-order mechanism originally proposed by Hartridge and Roughton (1925) is assumed, the reaction half-times of adult and foetal oxyhaemoglobins under these conditions are 0.015 and 0.021 seconds respectively.* The implication is clear: the molecules are protected by bound oxygen against oxidation and, under the given conditions, the oxygen binding of adult haemoglobin is more firm than that of foetal. This is confirmed by the fact that oxidation of foetal oxyhaemoglobin with sodium nitrite proceeds at twice the rate as that of the adult pigment, though in their reduced forms the haemoglobins again show no difference. Betke and Scholz (1958) have shown that the effect does not arise from differences in saturation of different haemoglobins at a given pressure.

A full account of Betke's work on the oxidation of animal haemoglobins, as well as the human haemoglobins A, F, C, and S (the last of which was investigated also by Betke and Greinacher (1955)), is to be found in an interesting recent paper (Betke, 1957). The reaction with ferricyanide appears to follow first-order kinetics, and, of the haemoglobins examined, heterogeneity is evinced only by human cord blood haemoglobin and by guinea-pig haemoglobin (Betke *et al.*, 1956a; Betke, 1957). The postulate of a summation curve due to two components is shown to give results for cord blood haemoglobin which are consistent with the results of alkali denaturation analysis. No differences are observed between haemoglobins A, C, and S. This is scarcely surprising since no differences in their oxygen-binding capacities have been discovered (Wyman and Allen, 1951). Betke finds that the oxidation of haemoglobins by nitrite ion is more complex, and advances evidence in favour of an autocatalytic mechanism (Betke, Greinacher, and Tietze, 1956b; Betke, 1957). On the other hand, Remmer and Meyer-Wilmes (1958) find that the reaction with ferricyanide is not stoichiometric, 2.3–2.9 equivalents being required per quarter-mol of haemoglobin. It is believed that another reaction also occurs, in which sulphydryl groups are oxidized.

Itano and Robinson (1956, 1957, 1958) have reported the oxidation of carboxyhaemoglobin to take place in successive steps, in each of which one haem iron atom is oxidized. Components have been resolved electrophoretically corresponding to these intermediates, and agreeing in their proportions with the results of spectrophotometric analysis.

Itano (N.A.S.-N.R.C. Conference, 1958, p. 151) has suggested that an absolute standard for the correlation of overall charge difference with relative migration rate for different haemoglobins might be available through comparison with such intermediates, which are known *a priori* to differ from each other by one charge unit. Reasoning of this kind indicates, for instance, that the charge difference between Hb-C and Hb-S is smaller than that between Hb-S and Hb-A.

Adsorption

The adsorption isotherms of various haemoglobins would have considerable interest, apart from their bearing on chromatographic behaviour. Only brief studies have been carried out, however, again by Betke and Greinacher, of the adsorption of Hb-A, Hb-F (1954a), and Hb-S (1955) on alumina, partly in connexion with the elimination of stromal materials from red cell haemolysates. Some differences were observed, notably that alumina will take up about 1.5 times as much foetal as adult haemoglobin.

Viscosity

Differences between the viscosities of solutions of Hb-S and Hb-A have been discussed by Allison (1957). The results of such work may be

*Complications arising from the use of sodium dithionite for the reduction of haemoglobin are described by Dalziel and O'Brien (1957). Roughton (in N.A.S.-N.R.C. Conference, 1958, p. 31) states that haemoglobin must not be exposed to dithionite for longer than a fifth of a second if it is not to undergo permanent change.

found to have a bearing on the structural differences between haemoglobin variants, in particular in terms of the disposition of groups responsible for intermolecular association and the role of the sulphydryl groups. No viscosity differences between the corresponding oxyhaemoglobins are found (Nakamura, 1955).

Sedimentation and Diffusion

A substantial difference between the sedimentation rates of foetal and adult haemoglobins in ultracentrifuge experiments was reported by Andersch, Wilson, and Menten (1944), who suggested on the basis of their results that Hb-F had half the molecular weight of Hb-A. Apart from the lack of support for such a hypothesis from any other experimental source, the sedimentation studies are not in agreement with the diffusion experiments of Taylor and Swarm (1949), who find that no difference exists between haemoglobins A and F. The ultracentrifuge technique has also been used to study dissociation equilibria in haemoglobin, and the results will be mentioned under another heading.

Crystal Structure

The characterization of haemoglobins in terms of their crystal structures has been carried out in recent years by Perutz and co-workers.

The crystallization of adult and foetal haemoglobins was first subjected to detailed study by Jope and O'Brien (1949) and later by Perutz *et al.* (1951), who also crystallized oxyhaemoglobin S. Under suitable circumstances these haemoglobins can give rise to tetragonal or orthorhombic systems. Single crystals of foetal carboxyhaemoglobin of known salt and water content have also been studied by *x*-ray methods by Zinsser and Tang (1951). Crystallographic interest has naturally centred on the reduced form of Hb-S, which exhibits notably different physical properties from other human haemoglobins (Perutz and Mitchison, 1950). (These will be discussed later under Hb-S.) Crystals of other haemoglobins have also been isolated, e.g., Hb-G and mixed crystals of Hb-A and Hb-G by Edington, Lehmann, and Schneider (1955), but no detailed analyses of structure have yet been published. Rossi-Fanelli, Antonini, and Mondovi (1957) comment on an anomalous crystal form associated with an abnormal methaemoglobin (apparently from Hb-M).

Absorption Spectra

The absorption spectra of all known haemoglobins have been examined with greater or lesser thoroughness.

The outstanding example of spectral deviation occurs in Hb-F, in which the tryptophan band is displaced toward a shorter wavelength (289.8 mμ) compared with Hb-A (291.0 mμ) and appears as a distinct absorption maximum rather than merely an inflexion (Jope, 1949; Beaven, Hoch, and Holiday, 1951). The analytical value of this observation is discussed in detail by Rich (1952) and by Beaven and Holiday (1952). The remainder of the visible and ultra-violet spectrum of foetal haemoglobin and its derivatives has been examined with great care (Lambrechts and Martin, 1951; Polosa, Motta, and Lo Turco, 1957), and it can be said with some certainty that no other significant differences in the positions of bands exist. Lambrechts and Martin (1951) examined the "Hufner quotients" for a number of wavelengths in the visible region of the Hb-F and Hb-A spectra, but found that intensity differences, if they existed, were less than 3%. Penati, Turco, Grandis, and Lovisetto (1955d), however, have reported that absolute intensity differences, although small, do exist in the ultraviolet, and present absorption curves of mixtures of adult and foetal haemoglobins in various proportions, which support their claims. The differences observed between pure adult and cord blood haemoglobins (*ca* 80% Hb-F) are of the order of 10 to 20% in the region of the tryptophan and tyrosine bands, with a maximum difference at 289 mμ. Some interesting curves are also given relating extinction coefficients at a number of wavelengths in the tryptophan band region with the percentage of Hb-F in a foetal-adult mixture.

Spectral deviations have been claimed in three other haemoglobins. Künzer and Drescher (1956) state that their pre-foetal haemoglobin, of intermediate alkali resistance, shows a tryptophan band at 289.8 mμ, and a sample supplied by Dr. Lehmann, and said by him to contain a new fast haemoglobin ("Bart's"), again, it may be noted, isolated from a mixture containing Hb-F, was examined with the logarithmic cam spectrograph and found to have a tryptophan band in the position characteristic of an Hb-A/Hb-F mixture in which the latter preponderates (Ager and Lehmann, 1958).

The Hb-M variant comes into a category of its own. This has been observed only as the methaemoglobin, which differs from normal methaemoglobin in features in its visible spectrum, in particular the presence of an absorption minimum, rather than a maximum, at about 630 mμ (Hörlein and Weber, 1948, 1951; Kiese, Kurz, and Schneider, 1956; Heck and Wolf, 1958; Rossi-Fanelli *et al.*, 1957; Gerald, Cook, and Diamond, 1957; Gerald, 1958). This observation, if accepted at its face value, is very extraordinary because it would seem to imply the existence of an aberration in the haem portion of the molecule. Hörlein and Weber (1948), as well as Kiese and Schneider (1955), claim to have shown, however, by splitting the haem-globin linkages and recombining the globin with haem derived from normal adult haemoglobin, that the abnormality is

in fact inherent in the globin. The significance of these observations will be discussed below.

Recently McCord and Gadsden (1958) reported small anomalies in extinction values in the visible spectrum of Hb-S. These anomalies, which were obtained from difference spectra of Hb-S and Hb-A derivatives, occurred, however, only at absorption maxima, at which the actual absorption values were greater by a factor of about 10 than that at the wavelength at which the solutions were matched. Under these conditions, errors in matching are greatly magnified.

Infra-red spectroscopy does not readily lend itself to the study of proteins: a survey of its application and shortcomings in this field is given by Sutherland (1952). A detailed examination of Hb-A in this region is described by Larson and McLaughlin (1955). As is to be anticipated, the comparison of haemoglobin spectra in the infra-red has not met with any great success. Studies on adult and foetal haemoglobins have been carried out by Greinacher, Greinacher, and Betke (1953) in the region between 2 and 15 μ, and on whole blood and haemolysates in various pathological conditions, including thalassaemia major, by Wegmann, Barthel, and Thewes (1957). In neither case were significant differences observed. Penati, Borello, Turco, and Lovisetto (1955b) have carried out similar investigations in the 2–11 μ region and have reported small but consistent differences between the ratios of peak intensities of the polypeptide bands at 7.7 and 8.03 μ in adult and foetal haemoglobins. Further investigation along these lines is unlikely to be profitable.

Differences Arising in Whole Blood

In the main, the behaviour of haemoglobins in whole blood is so heavily influenced by extraneous factors that it lies beyond the scope of this review. A few observations having preparative potentialities or implications shedding light on the physical or chemical specificity of a given haemoglobin species will nevertheless be briefly mentioned.

Centrifugation. — Bromberg, Rabinovich, Abrahamov, and Salzberger (1956) have recorded the interesting observation that the centrifugation of red cells of cord blood gives rise to a layering in which foetal haemoglobin occurs in higher concentration at the bottom than at the top. It may be possible to explain this phenomenon in terms of the results of Simon and Topper (1957) on the centrifugation of cells containing labelled haemoglobin. These workers found that a stratification occurred with the oldest cells at the bottom. Since at birth the rate of erythropoiesis of Hb-A is increasing whereas that of Hb-F is decreasing, so that the average age of cells containing predominantly the latter would be expected to be the greater, the layering in cord blood might be a manifestation solely of cell age and not of haemoglobin type.

Haemolysis Effects.—Although in general the haemolytic behaviour of a red cell is in no sense a function of the haemoglobin type that it contains, the occurrence of haemoglobin variants is frequently accompanied by altered fragility characteristics.

A large number of osmotic fragility studies have been made, and it seems likely that, on the basis of some of the results of such work, a fractionation of haemoglobins from mixtures occurring in blood samples could be achieved. Recent examinations of osmotic resistance of erythrocytes containing foetal haemoglobin have been made by Dettori (1957), by Astaldi, Tolentino, and Sacchetti (1955), and by Sjölin (1954). Erythrocytes containing Hb-S have been the subject of particularly thorough investigations in relation also to their increased mechanical fragility (Lange, Minnich, and Moore, 1951; Griggs and Harris, 1956). Osmotic fragility measurements have also been made on cells containing haemoglobins C (Spaet, Alway, and Ward, 1953), D (Chernoff, 1958), E (Chernoff, Minnich, and Chongchareonsuk, 1954), and H (Spaet *et al.*, 1953; Vella, 1957; Brain and Vella, 1958). The effect of cell age must again be borne in mind (Simon and Topper, 1957).

Immunological Specificity

The specificity of haemoglobins F and A in the precipitin reaction was demonstrated by Darrow, Nowakovsky, and Austin in 1940.

Subsequently the antigenic behaviour of these haemoglobins was examined by Vecchio and Barbagallo (1950), by Goodman and Campbell (1953), and by Chernoff (1953b), who claims by an immunological method to have shown that foetal haemoglobin is present to the extent of 0.05 to 0.5% in normal adult blood (Chernoff, 1953a), by Aksoy (1955), and by Sansone and Durando (1951). Recently Diacono and Castay (1957) have demonstrated specificity of Hb-F by a complement deviation method and have also examined the antigenic behaviour of haemoglobins S and C (1956a, 1956b). Other immunological work has also been carried out on Hb-S, but its characterization by this means is reported to be difficult (Goodman and Campbell, 1953). Summaries of the "double diffusion" technique in agar as applied to human haemoglobins, together with the results obtained, are given by Ruggieri and Marchi (1955) and by Chernoff (in N.A.S.-N.R.C. Conference, 1958, p. 179). An attempt has also been made to analyse human haemoglobins by immunoelectrophoresis (Boivin and Hartmann, 1958).

Genetic Criteria

One case can be cited in which the evidence for the existence of a haemoglobin variant rests very heavily on the genetic circumstances associated with its occurrence. This is the "fast" haemoglobin of Fessas and Papaspyrou (1957), which

was found in a child whose parents were suffering from thalassaemia and thalassaemia trait. Its behaviour in paper electrophoresis was only subtly different from that of haemoglobins I and J, but distinct from Hb-H. The genetic manifestations are consistent only with the behaviour of Hb-H (Motulsky, 1956). This being ruled out, the authors conclude that the new entity is distinct from haemoglobins I and J, and is a hitherto unknown form.

Structural Differences

Amino-acid Composition.—It is not proposed to analyse the results of the numerous terminal group and total amino-acid analyses which have been made on the human haemoglobins.

It must be noted that some of the discrepancies between the results of different workers seem to arise in vagaries in Sanger's dinitrophenyl technique. An examination, for instance, of the end-groups of Hb-A by Brown (1952) indicated in the first place two valine and 43 lysine ε-terminal groups, but with other, seemingly more reactive preparations of the dinitrofluorobenzene reagent further reaction occurred, giving in all some five valine residues. Rhinesmith, Schroeder, and Pauling (1957b) find that Hb-A has four N-terminal groups, all valine. Terminal groups have been examined by Masri and Singer (1955), applying the Sanger DNP method to haemoglobins A, C, F, and S, by Havinga (1953) working with haemoglobins A and S, by Schapira and Dreyfus (1954) on haemoglobins derived from subjects suffering from thalassaemia, by Huisman and Drinkwaard (1955) on haemoglobins A, C, F, E, and S, and by Huisman and Dozy (1956) on haemoglobins A, C, F, and S by digestion with carboxypeptidase.* The amino-acid nitrogen contents of haemoglobins S and A were shown by Schroeder, Kay, and Wells (1950) and by Dickman and Moncrief (1951) to be very similar.

Complete amino-acid analyses on haemoglobins F and A were carried out by van der Schaaf and Huisman (1955a), who obtained the curious result that the alkali-resistant fraction in the haemoglobin of sickle-cell anaemia is not the same as Hb-F, by Dustin, Schapira, Dreyfus, and Hestermans-Medard (1954), using globin derived from Hb-F, by Rossi-Fanelli, Cavallini, and de Marco (1955), by Huisman, Jonxis, and van der Schaef (1955) on haemoglobins A, S, F, and C, and by Jonxis, Huisman, van der Schaef, and Prins (1956) on Hb-E. The most interesting result which has accrued is the discovery of a large difference in the content of *iso*leucine, which is present in quantity only in Hb-F (van der Linden, 1950; Rossi-Fanelli *et al.*, 1954). Derrien and Laurent (1955a), for instance, find that Hb-F from cord blood has an *iso*leucine content of 1.94%, and that normal adult carboxyhaemoglobin contains 0.34%.

They find further that the so-called alkali-resistant fraction of normal adult oxyhaemoglobin contains 3.25% and the corresponding fraction of adult carboxyhaemoglobin 1.9% *iso*leucine. The presence of two types of alkali-resistant haemoglobin in adult carboxyhaemoglobin is inferred. The evidence, however, appears a little slender for so remarkable a conclusion. Other minor differences in composition are reported by Rossi-Fanelli, Cavallini, de Marco, and Trasarti (1955c). Recently a most interesting paper has appeared by the Rockefeller group of workers (Stein *et al.*, 1957), in which determinations of amino-acid compositions of haemoglobins A, F, S, and C are described, which are without doubt the last word to date in accuracy and reliability. The haemoglobins were isolated pure and free from minor components by electrophoresis on starch. *Iso*leucine was present only in the minutest traces except in Hb-F. The highest content in the other haemoglobins was 0.45 of an *iso*leucine residue per molecule of Hb-C. It is clear, therefore, that the *iso*leucine content of the haemoglobins examined, other than Hb-F, arises from impurities. Moreover, a simple calculation shows that if this impurity, when detected in normal adult haemoglobin, were Hb-F, the *iso*leucine content is such as to indicate its presence in quantities from 10 to 25%. It is to be concluded categorically, therefore, that the impurity in question is not Hb-F. It is conceivable that the *iso*leucine derives from the colourless component described by Derrien (1957; Derrien, Laurent, and Borgomano, 1956) and associated with the so-called alkali-resistant fraction of normal adult haemoglobin, said by him to contain some 4% *iso*leucine. Rhinesmith *et al.* (1957a) reported that heterogeneity in normal adult haemoglobin disappeared on crystallization, but Allen *et al.* (1958) found that heterogeneity on ion exchange columns remained, though the *iso*leucine content of the protein was unchanged by crystallization (but cf. Rossi-Fanelli *et al.*, 1955b), though different portions of the column eluates contained widely different amounts of *iso*leucine, and some indeed none at all. The drastic effect which impurities or minor haemoglobin components can have on amino-acid composition is thus evident.

Schroeder and Matsuda (1958) have also found that Hb-F and Hb-A contain equal numbers of glycyl residues, as well as valyl N-terminal groups. It is suggested that the N-terminal sequence valyl-leucyl occurs in both Hb-A and Hb-F, and that these proteins may each have one identical chain containing such a sequence, though differing in the other chain which terminates in glycine in Hb-A and a valyl-histidyl-leucyl sequence in Hb-F.

A significant development in the molecular analysis of haemoglobins has been developed by Ingram (1957; Hunt and Ingram, 1958b), who has devised

*The results of these workers for Hb-A are not altogether in accord with the C-terminal amino-acid determination of Kauffmann and Boettcher (1958), who find that, using hydrazine, two C-terminal histidine groups are released from Hb-A as against one if carboxypeptidase is used. An interesting discrepancy between determinations in haemoglobin and on the isolated globin was noted and is ascribed to the release of free amino-acid in the presence of haem.

means of breaking up the haemoglobin into short-chain peptides, which are examined and identified by two-dimensional paper electrophoresis and chromatography. The composition of these peptides gives a first indication of the order in which amino-acid residues occur in the protein, and the interesting result has emerged that haemoglobins S and C differ from Hb-A in the identity of only one residue.

Hunt and Ingram (1958a) have gone a stage further in explaining the molecular nature of haemoglobins, and have digested the trypsin-resistant "core" of haemoglobins A and S with chymotrypsin. The "core" comprises a considerable portion of the protein, and is poor in lysine and arginine, but rich in aromatic amino-acids, and those with long non-polar side chains. No differences between Hb-A and Hb-S were found.

The method of "differential titration" on filter paper of Scheinberg *et al.* (1954), already alluded to, is interpreted by these authors in terms of free carboxyl groups, but the inconsistency of their results—most notably in the case of Hb-C—with the results of end-group analyses, leaves the validity of their reasoning open to doubt. It has recently been reported (Benzer, Ingram, and Lehmann, 1958) that analysis of three different Hb-D samples by tryptic digestion has shown them to have different compositions. It has been previously pointed out (Vella, Wells, Ager, and Lehmann, 1958) that where a given variant has been obtained from a number of ethnically distinct sources, the identity may be only apparent, resulting from a similarity in electrophoretic and/or chromatographic behaviour. The conclusion then presents itself that if the methods used are reliable there are indeed a great many haemoglobin variants. The number of possible arrangements of units in the protein chains which could give rise to functional haemoglobins might be expected to be very large indeed.

Sulphydryl Groups.—The relation between the free sulphydryl groups in the haemoglobin molecule and the nature of the oxygen dissociation curve (Riggs, 1952) has been mentioned.

Ingbar and Kass (1951) carried out amperometric titrations of haemoglobins A and S and found the former to contain two and the latter three accessible sulphydryl groups. More recently, Ingram (1955) has examined Hb-A and has found by titration with silver nitrate in ammonia that four sulphydryl groups are present. Partial denaturation leads to an increase in the number of titratable groups in Hb-A to eight (Benesch, Lardy, and Benesch, 1955; Ingram, 1955). These results are not confirmed by Allison and Cecil (1958), who describe amperometric titration with mercuric chloride and phenylmercuric chloride, and by making certain assumptions about the nature of the binding of these reagents with the protein arrive at the conclusion that native Hb-A contains two, and denatured Hb-A six, reactive sulphydryl groups. The increase in the number of titratable groups can arise from the mildest treatment, such as the elevation of the temperature to 38° C., as shown by Murayama (1957a, c, 1958), except in Hb-F (Murayama, 1957b; Hommes, Santema-Drinkwaard, and Huisman, 1956). It is clear that this behaviour is governed predominantly by steric factors. The configurational implications are discussed by Murayama (1957a). (For criticisms of his deductions, see N.A.S.-N.R.C. Conference, 1958, pp. 238–252.)

Hommes, Dozy, and Huisman (1958) have examined Hb-F and find that, of the six half-cystine residues present, four are in the form of reactive sulphydryl groups. After reduction with thioglycollic acid, the protein is found by electrophoresis to have been split into two components. The presence of two different polypeptide chains joined by one or more disulphide bridges is inferred.

Stein *et al.* (1957), working with their electrophoretically purified haemoglobin samples, find four to five sulphydryl groups per molecule of haemoglobin, except in Hb-F, for which they find a value of 3.4, and they state also that determinations of the half-cystine content of the haemoglobin examined agreed with the amperometric titrations. These authors have observed in addition that treatment with sodium sulphite does not give rise to an increased number of titratable sulphydryl groups. The results of titrations of haemoglobins which have not been purified by electrophoresis, that is to say, in the condition in which they have presumably been examined by other workers (Hommes *et al.*, 1958, etc.), exhibit variations from sample to sample, which are largely removed by electrophoresis. It is concluded that there are present impurities, rich in sulphydryl groups, which are largely separated by electrophoresis on starch.

The condition of the sample is also of importance. It is known that sulphydryl groups are easily oxidized not only by the ferricyanide ion and other reagents (Remmer and Meyer-Wilmes, 1958) but also by molecular oxygen. This has been shown by Tsen and Tappel (1958). Taylor (1955) has shown that ferricyanide oxidation with consequent transformation of sulphydryl groups gives rise to an effectively new molecular species with different solubility and electrophoretic mobility. The results of most of the older determinations of active sulphydryl groups perhaps demand re-examination in this light. Kajita (1956) used an interesting method for the evaluation of free sulphydryl groups, in which oxyhaemoglobin was oxidized with potassium ferricyanide. Aliquots were taken during the reaction and analysed for total consumption of reagent and methaemoglobin formed. By subtraction this gave a value of only one free sulphydryl group per molecule of native Hb-A at a *p*H of 5.5 to 8.5. This was increased to 2 to 2.5 when the *p*H was raised to 9.5, and the addition of protein "perturbators," such as benzoic acid in high concentration, gave a further increase to three groups.

It should be noted also that results obtained by different techniques for the estimation of reactive

sulphydryl groups are often not consistent (Hommes and Huisman, 1958). An evaluation of the methods available is evidently necessary.

FACTORS INFLUENCING THE BEHAVIOUR OF HAEMOGLOBIN

A number of properties of haemoglobin will be discussed which, it is thought, may have an important influence on the results derived from the techniques already outlined, and which have so far received little attention in the literature.

Dissociation of Haemoglobin

The first of these properties is the tendency of haemoglobin to dissociate under certain conditions into sub-molecules. This has been carefully investigated by Field and O'Brien (1955) and its effect on boundary spreading in the course of electrophoretic migration is discussed by Field and Ogston (1955). Field and O'Brien, on the basis of sedimentation and diffusion measurements on carboxyhaemoglobin, conclude that the behaviour is independent of *p*H over a range of about 6 to 11. Between *p*H 3.5 and 6 a rapid reversible dissociation into fragments of about half the molecular weight of the haemoglobin molecule occurs, and at lower *p*H, or on prolonged exposure to *p*H 5, this is succeeded by an irreversible denaturation. The dissociation is promoted by dilution as well as by reduced *p*H, and the presence of extraneous substances such as urea.

In 1953 Derrien and Reynaud examined adult carboxyhaemoglobin by free boundary electrophoresis, first in a phosphate buffer at *p*H 6.5 and ionic strength 0.1, and observed the presence of two components, one of them in small quantity. This agrees as far as it goes with Hoch (1950), who, however, observed further small components, of which more will be said later. In cacodylate under similar conditions, however, Derrien and Reynaud observed no less than six components, one of which was a major one. Now this could be related to the artefacts described by Woods (1958) in relation to bovine serum albumin and already referred to, but Itano (1956) has found that the heterogeneity in this case disappears if the cacodylic acid used in the buffer is first recrystallized from alcohol. The implication is that impurities are present which bring about the denaturation of the haemoglobin, with consequent onset of heterogeneity. It would be difficult, however, to explain in the same terms the observations of Shavit and Breuer (1955), on normal adult carboxyhaemoglobin in cacodylate buffer at *p*H 6.5, that more and more components successively appear as the ionic strength is lowered from 0.1 to 0.005, that is to say, as the concentration of cacodylic acid, with or without impurities, is decreased. They also find that a rise in temperature from 1.5 to 20° likewise gives an increase in heterogeneity.* It is clear that these phenomena might well have a profound bearing on the interpretation of data from free boundary electrophoresis experiments. It is also interesting to note, *à propos* of Itano's observation, the drastic effect which the presence of a minute proportion of an impurity can have on the behaviour of haemoglobin. It might be mentioned that the electrophoretic identification of intermediate stages in the oxidation of carboxyhaemoglobin to methaemoglobin by Itano and Robinson (1956, 1958) was carried out at *p*H 6.85 and the very low ionic strength of 0.01, albeit in phosphate buffer. A number of examples of the characterization of individual haemoglobins by free boundary electrophoresis will be given later.

The possibility of a dissociation equilibrium involving the recombination of sub-molecules derived from parent molecules of different species is also not to be entirely discounted, though recombination of the related sub-molecules would be expected to occur preferentially. Crosswise recombination is believed to occur by Reiner, Moore, Lang, and Green (1942), who observed two fractions in bovine globin at low *p*H, which gave identical absorption spectra, apart from some differences in extinction values. These globins were separable electrophoretically, and also differed in their acid-binding capacity. Their relative proportions indicated that they did not derive from two haemoglobins, and the authors suggested that they arose from recombination of sub-molecules. Moore and Reiner (1944) subsequently extended these observations to the globins of other animals and man. The relative proportions of the fast and slow components changed with *p*H, though with prolonged electrophoresis heterogeneity occurred at any *p*H. The heterogeneity was confirmed by ultracentrifuge experiments at low *p*H, and the Svedberg constants for the two components thus obtained agreed well with those of the two fractions isolated electrophoretically which were

*It has also been claimed that dissociation of part of the oxyhaemoglobin in solutions at *p*H values near the iso-electric point occurs in the presence merely of sodium chloride (Benhamou and Weill, 1956, 1957), though the method used, that of light scattering, is of doubtful applicability. Indeed, other workers (Putzeys *et al.*, 1955) using the same technique find that, so far from dissociation, aggregation occurs under these conditions.

themselves homogeneous. The disintegration of horse haemoglobin into four subunits of roughly equal mass but different mobilities was also shown by Reichmann and Colvin (1956). On the other hand, Haug and Smith (1957) and Smith *et al.* (1957) have evidence that the four subunits are of two types, differing in amino-acid composition. It may also be mentioned that Lewis (1954) and Hughes (in N.A.S.-N.R.C. Conference, 1958, p. 62) have observed that half the haem groups of haemoglobin are more easily removed than the remainder.

Steinhardt (in N.A.S.-N.R.C. Conference, 1958, p. 49) gives reasons for supposing that human haemoglobin is dissociated further, into four fragments, at *p*H 2.8 at 0° C.

Haemoglobin Binding

The problem of impurities, which is raised by Itano's observation on the heterogeneity induced by impure buffer salts, must now be pursued.

In 1954 Anderson and Griffiths recorded their observations on haemoglobin samples from subjects carrying the sickle-cell trait. The mobilities found for the components in free boundary electrophoresis in phosphate buffer at *p*H 7.5 and ionic strength 0.1 were not constant. Over nine samples the mobilities of the Hb-A component varied between -1.2 and -2.4×10^{-5} and that of the Hb-S component between -1.7 and -3.8×10^{-5} cm./sec./v./cm. Similar variations were seen on paper. These results, it was claimed, were not artefacts because the figures from a given sample were repeatable, and it was shown spectrophotometrically that methaemoglobin was in all cases absent. Anderson and Griffiths suggested on the basis of these results that apparent heterogeneity in haemoglobins might in fact be due to binding of the haemoglobin with other blood constituents, in particular, haptoglobins and nucleic acids. Mixtures of haemoglobin with nucleic acids were accordingly made, dialysed against buffer, and run in a Tiselius electrophoresis apparatus. The velocity of the haemoglobin was altered and a new peak observed. Similar results were obtained with carbonic anhydrase and with globin (though in the latter case the result of threefold heterogeneity is incompatible with the successful use of globin in admixture with haemoglobin to prevent adsorption of the pigment in paper electrophoresis as reported by Hoch and Barr in 1956). It was also found that, if samples of sickle-cell trait haemoglobin were dialysed before electrophoresis, two new components of intermediate mobility appeared, which were ascribed to dissociation and altered recombination of the complexes. Though the inferences of Anderson and Griffiths are perhaps a little extravagant, their work nevertheless deserves notice, since binding with various materials in the haemolysates does occur. This would be expected to be particularly severe where whole blood haemolysates are used, or devices are employed such as the partial separation of serum proteins by lead subacetate. That such a process should be recommended for the preparation of haemoglobin solutions for electrophoresis (e.g., Tompkins, 1955) is indefensible, and indeed any results of experiments in which whole blood haemolysates or extracts from unwashed erythrocytes or homogenized clots (Schneider, 1956) are used are open to criticism. It is known that some serum proteins combine most tenaciously with haemoglobin. Foremost among these are the haptoglobins, which have been described by Jayle *et al.* (1952), Jayle and Boussier (1955), Wieme (1953), Reich (1956), and other authors. Binding with these substances, which vary with the individual (Smithies, 1955), substantially modifies the electrophoretic mobility. Tuttle (1955) found that these substances were absent in the blood of newborn children. It is also known that haem groups can combine with the albumin fraction of human serum (Keilin, 1944; Rosenfeld and Surgenor, 1950; Schwerd, 1958; etc.) to give methaemalbumin, and interaction with globulin fractions has also been described (Schwerd, 1958). Binding of serum proteins can vitiate the results of alkali denaturation experiments (Künzer, 1955), and it is noteworthy that the colourless proteins separated by Derrien (1957; Derrien *et al.*, 1956) from Hb-A were found to be associated with the "alkali-resistant" portion. A spectroscopic study has been made by Betke, Greinacher, and Leber (1954), who make the very feasible suggestion that the so-called alkali-resistant component which has been reported from time to time to be present in normal adult haemoglobin (Singer *et al.*, 1951; Huisman *et al.*, 1955; Künzer, 1955, 1957a, etc.) is in fact a complex between the pigment and serum protein. The severe interference in Hb-F estimations caused by the presence of plasma is described by Rappaport, Eichhorn, Konforti, and Roessler-Wolfson (1957). Iversen and Larsen (1956) found that whole blood haemolysates from patients in various anaemic conditions gave alkali denaturation curves of the type associated with Hb-F. This effect vanished when haemoglobin solutions

prepared from washed erythrocytes, and separated from stromal materials, were used. The effect is evidently due to binding with substances deriving from the stroma or plasma. Binding with stromal material, presumably lipoproteins, was shown to occur by Ponder (1952), who found that the complexity of the paper chromatography pattern of adult haemoglobin increased when stromal material was present. Binding of this kind was also demonstrated by Dervichian, Fournet, Guiner, and Ponder (1952b), who examined the orientation of haemoglobin molecules on stromal fragments. It has also been noted (Baar and Hickmans, 1941) that a spurious alkali-resistant component appears in haemolysates which have been prepared with the aid of saponin. A further potential source of minor alkali-resistant components is the presence of carboxyhaemoglobin from endogenously formed carbon monoxide (Sjostrand, 1958). An interesting source of a similar phenomenon is contact with rubber (stoppers, etc.), which has been described by Raper (1957).

The binding of haemoglobin with nucleoproteins is known to be possible (Nelson, 1958), and such substances are present in leucocytes and reticulocytes (Holloway and Ripley, 1952). Various other phosphorus compounds occur in mature erythrocytes (Rottino, Hoffman, and Albaum, 1952; Kutas and Stützel, 1958; etc.). Moreover, Havinga (1953) has shown that Hb-S, unlike Hb-A, has a small phosphorus content, which may be due to bound nucleic acid. Again, Breuer, de Vries, Peket, and Matoth (1957) find that the onset of heterogeneity effects in free boundary electrophoresis occurs earlier, that is to say, at higher ionic strength and lower temperature, in haemoglobin solutions from subjects in various pathological conditions, such as spherocytosis, not associated with abnormal haemoglobins. These authors suggest that this behaviour may be governed by protein constituents in the erythrocytes which remain in the haemolysate and which may be absent in pathological disorders.* It is not inconceivable that the minor components of normal adult haemoglobin which have been observed by a large number of authors (see below) may come about in this way, especially when the decrease in the proportion of secondary component in solutions on standing, as observed by Berry and Chanutin (1957, 1958), is borne in mind. Changes in the heterogeneity pattern associated with microcytosis (Modiano, 1957; Silvestroni and Bianco, 1957), etc., and the curious small components of Masri *et al.* (1958) may also be noted. Some indication in this direction is also provided by the remarkable observations of Cabannes, Raffi, and Boineau (1957a) on subjects in a family suffering from a haemolytic disorder. These authors claim to have separated an A_2 component by starch slab electrophoresis which is present in three members of the family to the extent of 21, 27, and 14% respectively. It is alleged to have been differentiated from Hb-E by its electrophoretic behaviour at *p*H 6.5. The extraordinary observation by Cook and Morrison (1956) that their minor component isolated from cord blood haemoglobin, running on the columns in the same position as A_2, is completely resistant to alkali under conditions where foetal haemoglobin itself is labile provides a further pointer.

The separation of colourless proteins from various haemoglobin solutions will be seen to have a further bearing on this problem and on the subject of electrophoretic mobilities in general. The content of material other than haemoglobin in the erythrocyte has been closely studied. Stern, Reiner, and Silber (1945) give as the main contents of the non-nucleated erythrocyte 87–95% haemoglobin, 5–12% of other proteins, 0.6% lecithin, about 0.4% cholesterol and varying traces of inorganic substances.* These constituents would be expected for the most part to remain in solution when the cells are haemolysed and centrifuged. The presence of serum proteins and possibly bilirubin in the haemolysate even from washed cells is not to be excluded, since the adsorption of such substances on the stroma (in the same way as haemoglobin is known to be adsorbed (Ponder, 1951)), followed by desorption on haemolysis, is possible. Certainly very little serum protein would be needed to give rise to the spurious effect of a perceptible proportion of alkali-resistant haemoglobin in normal adult blood (Künzer, 1955; Iversen and Larsen, 1956). These cell constituents would be either bound or else in any case too large to be removable by dialysis. Their presence in a haemoglobin solution can be detected electrophoretically and sometimes by other means. Muller and Zijlstra (1957), for instance, found that when they attempted to prepare solutions of known methaemoglobin

*The possibility is mentioned that a tendency for the haemoglobin molecules to dissociate particularly readily may be responsible for the haemolytic disintegration of the erythrocytes in some pathological conditions.

*A more detailed account of erythrocyte constituents is given by Behrendt (1957).

contents for analytical purposes by mixing methaemoglobin and oxyhaemoglobin solutions the proportion of the former was reduced, an effect attributable only to the presence of the methaemoglobin reductase enzyme in the solution. A non-haem coloured protein can be separated, though with some difficulty, by electrophoresis (Lonn and Motulsky, 1957; Masri *et al.*, 1958). Lonn and Motulsky (1957; N.A.S.-N.R.C. Conference, 1958, p. 216) believe this to be methaemoglobin reductase. A putative methaemoglobin reductase has also been separated by Morrison (in N.A.S.-N.R.C. Conference, 1958, p. 167) on ion exchange columns. The separation of non-haemoglobin proteins has also been described by Giri and co-workers (Giri and Pillai, 1956; Giri *et al.*, 1958), who used electrophoresis on agar followed by amido-black staining. It is also a matter of common observation that a clear centrifuged haemoglobin solution will often precipitate stromal material on large dilution or *p*H or temperature change (Ponder, 1942).

The bearing of the presence of dissolved matter of one kind or another on the nature of the solubility curve has already been touched on in discussing the work of Karvonen and Leppänen (1952b), and the lecithin constituent of the erythrocytes suggests itself as a likely protective colloid. The efficacy of substances of this kind as emulsifying agents is well known. It would be surprising if the presence of these various impurities were not in fact reflected in the solubility curves. Another relevant indication is the fact that, whereas sickling in erythrocytes containing Hb-S occurs when the proportion of this pigment is 7%, for gelling in solution 10% is required. This has been shown by Singer and Fisher (1953), who conclude that the solubility is governed partly by intra-erythrocytic substances.

The work of Roche, Derrien *et al.*, already mentioned (Roche and Derrien, 1951, 1953; Roche *et al.*, 1952, 1953, 1954), could well bear re-examination in this light. Moreover, Betke (1957) has found that the course of oxidation of haemoglobin solutions with sodium nitrite depends markedly on the manner in which the solution has been prepared, whether by simple haemolysis of the red cells, by haemolysis followed by adsorption of extraneous material, or by haemolysis and adsorption followed by dialysis. It is also found that oxidation proceeds differently when crystallized haemoglobin is used (Remmer and Meyer-Wilmes, 1958). Another possible interpretation of this phenomenon will be discussed below.

The separation of colourless proteins from haemoglobin in electrophoresis experiments has been achieved on a number of occasions. Stern *et al.* (1945) isolated a colourless, opalescent component from normal adult haemoglobin by free boundary electrophoresis. This constituted some 2–5% relative to the haemoglobin, and was shown by the Tyndall effect to consist of large particles. It may therefore be a macromolecular protein, a lipoprotein, or stromal material in general. Its mobility is stated to be reasonably consistent with the latter explanation. Giri and co-workers (Giri and Pillai, 1956; Giri *et al.*, 1958) report that three non-haem protein constituents are separable by electrophoresis on agar of red cell haemolysates, and Allen *et al.* (1958) find one such substance associated with one of the haemoglobin constituents fractionated on their ion exchange columns. Slow-running, pale-coloured substances have been isolated on starch blocks by Masri *et al.* (1958) (associated with the "A_4" haemoglobin component) and by Kunkel *et al.* (1957). On paper also, peaks are observed by densitometry in the trail of the haemoglobin zones (Goldberg, 1957), and de Traverse *et al.* (1958) find that a slow moving band is clearly visible after amido black staining. Derrien *et al.* (1956) found a colourless protein present to the extent of 1.3 to 1.5% in normal adult haemoglobin solutions. This is said to be associated with the so-called alkali-resistant fraction of the haemoglobin, separable from it by paper electrophoresis and constituting 43% and 24% of the alkali-resistant fraction when separated respectively from the oxy- and carboxy forms. This result is compatible with the possibility, as outlined above, of complex formation with protein materials leading to an apparently alkali-resistant pigment. The behaviour of this system as described by the above-mentioned authors suggests that an equilibrium may be involved. A minor component of unknown nature has also been observed in Tiselius electrophoresis of mixtures containing Hb-S and Hb-C (Sturgeon, Itanø, and Valentine, 1952; Singer, Kraus, Singer, Rubinstein, and Goldberg, 1954).

Zinsser (1952) has found that the presence of the caprylate ion not only alters the mobility of Hb-F in a free boundary apparatus substantially but also caused a colourless protein to be displaced from it. This may well be a bound component, since Zinsser has shown that it does not arise from haem displacement. (It should be noted, however, that fatty acid ions can behave

as denaturants.) Another similar observation may also be mentioned here, viz., the appearance of a precipitate when solutions of haemoglobins K and N are diluted with a cacodylate buffer (ionic strength 0.1) (Robinson, Zuelzer, Neel, Livingstone, and Miller, 1956). Goldberg (1957) notes that in paper electrophoresis the amount of material remaining at the point of application is always greater when abnormal haemoglobins are present. The profound effect of extraneous matter in haemoglobin solutions is emphasized very strongly by the results given in the paper already referred to (Stein *et al.*, 1957) by the Rockefeller group of workers, who found that the *iso*leucine content observed hitherto in Hb-A was in fact due to an impurity which was not Hb-F. Differences between crystallized and uncrystallized haemoglobin have also been noted (Rhinesmith *et al.*, 1957a; Rossi-Fanelli *et al.*, 1955b), and Allen *et al.* (1958) have obtained fractions from their ion exchange columns which differ widely in their *iso*leucine content. It is possible that substances varyingly rich in *iso*leucine are bound to some of the haemoglobin, particularly when the differences (if real) between the *iso*leucine contents of oxy- and carboxyhaemoglobin A (Derrien and Laurent, 1955a; Allen *et al.*, 1958) are considered, and that these complexes separate on columns. The particularly high content of *iso*leucine in the trailing edges of chromatographic and electrophoretic zones may be related to the presence of non-haem proteins in these regions in electrophoresis (Giri *et al.*, 1958). Still more impressive is the evidence (Stein *et al.*, 1957) that large variations in the number of titratable sulphydryl groups arise from foreign substances which are rich in sulphydryl.

It is also interesting that the incubation of proteins with amino-acids under conditions similar to physiological ones causes binding, a good deal of which is due to adsorption but some of which is firm, the amino-acid being inseparable by dialysis (Cornwell and Luck, 1958). The amount of such binding is a function of the *p*H and also of the amino-acid concentration in the system. Since it is known (Iyer, 1957, 1958) that in some pathological conditions the level of free amino-acids in erythrocytes is increased, it would not be surprising if haemoglobin-amino-acid complexes were to be found in haemolysates. These would behave presumably as separate species. It will be seen, therefore, that the degree of purity of haemoglobins is of the first importance in assessing their behaviour in any respect and that bound or unbound impurities can give, and no doubt have given, rise to a great many specious results. The apparently anomalous haem-protein ratio of the A_2 component, as examined by Kunkel and co-workers (Kunkel *et al.*, 1957; Ceppellini, 1956), might be regarded as support for the binding hypothesis, as indeed might the observation that the elution curves of Allen *et al.* (1958) from ion exchange columns appear to give different proportions for the minor component of Hb-F, when the concentrations are measured respectively at 280 and 415 mμ. Derrien *et al.* (1956) take a similar view of their minor component.

The nature of the inorganic ions in the erythrocyte, and subsequently in the solution, though their concentration would be small, might well have an effect not only on the solubility curve (Karvonen and Leppänen, 1952a), but also on the electrophoretic behaviour, which is known to depend in large degree on the ions which are present (e.g., Shavit and Breuer, 1955; Morrison *et al.*, 1954; Shooter and Skinner, 1955). The effect of various ionic species on the behaviour of the haemoglobin has been all too little studied. Examinations of rather limited scope have been made by Morris and Wright (1954) and by van Os and Möller (1958), and Scheler (1957) has discussed in great detail the influence of various ions on equilibra in methaemoglobin solutions. Amongst other conclusions which emerge is the likelihood that a part of their action is due to colloidal stabilization. In general it may be said that interactions of haemoglobin with inorganic ions or other dialysable constituents is by no means as problematical as binding with proteins and like materials* though the effect on oxygen dissociation has already been dealt with. The nature of the dialysable substances, on which the shape and position of the oxygen binding curve so heavily depend (Geiger, 1931; Allen *et al.*, 1953; Becklake *et al.*, 1955), is obscure, though some speculations have been made by Hořejší and Komárková (1958). Their further investigation would be of some interest, especially in relation to the striking differences pointed out by Riggs and Tyler (1958), in the slopes of the graphs relating log (oxygen tension at half saturation) and log (body weight) for a number of mammals.

*One case at least is known, however, in which binding with an ionic species is of a very firm nature. Remmer (1958) has found that methaemoglobin, prepared by the use of ferricyanide, contains twice as much iron as is indicated by the pigment concentration as measured spectrophotometrically. The excess iron is not removable by dialysis and Remmer gives reasons for supposing that it is in the form of ferrocyanide co-ordinatively bound to the globin.

These are in contrast with the minute species differences between mammalian haemoglobins, as reflected in their absorption spectra, etc.

Interaction between Haemoglobins

Interactions between different haemoglobins have often been observed, and are in many cases of a quite firm nature. Such interactions can result in anomalous migration velocities in free boundary or zone electrophoresis and in incomplete separation.* The work described above on the results of column fractionation of haemoglobins A and F is presumably a similar manifestation.

The failure of haemoglobins A and F to separate on paper and various other media must arise from an interaction between the two proteins. Itano *et al.* (1956) suggest that this interaction may cause the trailing observed on paper, though this is perhaps rendered unlikely by the work of Hoch and Barr (1956) with haemoglobin/globin solutions mentioned above. Instead of separation on paper a single zone only is to be seen, the speed of migration of which depends on the proportion of Hb-F in the mixture, and has in some cases been used to estimate this proportion (Gatto and La Grutta, 1955a). Zinsser's measurements on the migration of haemoglobins derived from foetal blood under free boundary conditions showed that the younger the foetus from which the haemoglobin had been drawn, the faster the rate of migration of the solitary haemoglobin zone at *p*H 6.98 in phosphate buffers of ionic strengths between 0.08 and 0.15 (Zinsser, 1952). Further reports of modified mobilities of haemoglobins (which might well be attended by only partial separation) have been made: Edington *et al.* (1955) find that the electrophoretic mobility on paper of Hb-G varies with the other haemoglobins present, and Vella *et al.* (1958) report that the migration of Hb-A on paper is elevated in the presence of Hb-J. Bergren *et al.* (1954) have noted the interaction between haemoglobins C and S, whereby Hb-S migrates more slowly in the presence of Hb-C, and Silvestroni and Bianco (1957) find that the migration velocities of the minor components of Hb-A are changed in the presence of Hb-C.

A striking example of mutual interaction is given by Ramirez and Dessauer (1957a, 1957b) and Dessauer, Fox, and Ramirez (1957), who have subjected turtle haemoglobin to free boundary electrophoresis. This haemoglobin consists of two species, one fast and one slow, having identical spectra; they were fractionated by a continuous electrophoresis method. It was found that in equimolar mixtures of the two carboxyhaemoglobins at *p*H 7.4 the mobility of the slow component was no less than twice that of the pure slow component under the same conditions (-4.0 as against -2.0×10^{-5} cm./sec./v./cm.) whereas that of the fast complement was decreased (-3.6 as against -3.9×10^{-5} cm./sec./v./cm.). More examples exist, but the ones cited should give sufficient indication that surprising results which are open to misinterpretation can be obtained in electrophoresis of haemoglobin mixtures. Mutual interaction of haemoglobins or interaction with other substances would certainly affect the subtle balance of distinction between many haemoglobins by paper electrophoresis, e.g., the "fast" haemoglobins as described by Cabannes, Sendra, and Buhr (1957b).

Shooter *et al.* (1958) have noted that in the resolution of haemoglobins of closely similar mobilities, whether by zone or free boundary electrophoresis, the apparent amount of the leading component is increased and that of the slower diminished. (They suggest that the non-resolution of the so-called A_3 component in the starch block separations of Kunkel *et al.* (1957; Kunkel and Bearn, 1957) is a consequence.) Interaction resulting in imperfect fractionation is thus indicated. It is suggested that similar effects occur in the fractionation of haemoglobins by ion exchange chromatography (see above). Interactions of a different kind also arise in the measurement of solubility when solid solutions occur. Mixtures of haemoglobins F and A display such effects (Itano *et al.*, 1956). An interesting case is the solubility of Hb-S in its reduced state, which is substantially less if haemoglobins A, C, and F, as well as other proteins such as serum albumin, are present (Singer and Fisher, 1953). Allison (1957) has investigated the aggregation of Hb-S in solutions of the reduced form by the measurement of viscosity, and his results lead him to the view that mixed polymers are readily formed by Hb-S with Hb-C, less readily with Hb-A (that is to say, the required proportion of Hb-S is greater), and not at all with Hb-F. A study of gelling in Hb-S solutions containing also haemoglobins A, F, or C has been made by Singer and Singer (1953), Hb-C being found to have the strongest influence on solubility. It may

*Spurious fractionations due to interactions in protein mixtures in general have been reported (e.g., Granzer, 1958). They are said to be due essentially to hydrogen bonding and can be eliminated by the addition of hydrogen bond-breaking substances. There is, however, considerable risk of irreversible denaturation.

be noted that interaction with solid solution formation would be expected to occur only in closely similar chemical species. Itano (1953a) observes that such behaviour occurs with a mixture of, for instance, horse and donkey haemoglobins, but not in haemoglobins of less closely related species (*cf.* also Bangham and Lehmann, 1958).

Effects from Denaturation or Modification of the Haemoglobin Molecule

Jope (1949) examined the absorption spectra in the visible and Soret regions of oxyhaemoglobins which had been exposed for varying periods to denaturating agents, in particular urea. The interesting results emerged that in the early stages of attack the Soret band was slightly modified, with a shift towards shorter wavelengths, whereas the visible bands remained unchanged. The possibility presents itself that in the denaturation process intermediates are formed, which may be regarded as slightly modified haemoglobins, having presumably the essential properties of a native haemoglobin, but behaving analytically as discrete molecular species. Jope's work was carried out under conditions not suited to accurate intensity measurements in the visible region. The authors have attempted to confirm Jope's results with the aid of more accurate optical methods, using in the first instance alkali rather than urea as the denaturing agent. A shift in the Soret peak was indeed found to occur. The peak absorption for oxyhaemoglobin is at 414 mμ, and that of alkaline haematin brought to about *p*H 7 is at 411 mμ, whereas a sample of oxyhaemoglobin exposed briefly to the action of alkali and neutralized has a maximum at 408 mμ. It follows that this solution is not a mixture of oxyhaemoglobin and alkaline haematin generated by denaturation of a portion of the native protein. It was also found that no perceptible changes in the position of the visible bands accompanied this modification, though their persistence was somewhat altered.* The question now arises whether the spectral shift is due to the formation of methaemoglobin as the first reaction intermediate, for this would be consistent with the change in persistence in the visible region. This possibility is being investigated.

A study was made by Banaschak and Jung (1956) of the action of urea on methaemoglobin. Its first effect is the formation of a product with a characteristic absorption band at 534 mμ at *p*H 7, shifting in more acid solution to 537 mμ with the Soret peak at 413 mμ, but substantially diminished in intensity. This product forms a cyanide but not an azide, and may well be regarded as irreversibly denatured, since removal of urea leads to immediate precipitation. On the other hand, reduction with dithionite gives a pigment with a band at 557 mμ, which is nevertheless not to be identified with reduced haemoglobin on account of the low intensity and the position (424 instead of 429 mμ) of the Soret band. Treatment with oxygen or carbon monoxide, however, leads to products whose spectra are very nearly identical with those of oxy- and carboxyhaemoglobins. Moreover, when the oxy-derivative is oxidized it gives a substance having a spectrum closely similar to that of methaemoglobin, and combining readily both with the cyanide and azide ions. This provides a striking example of a modified haemoglobin, and provokes interesting speculation on the factors governing the coupling of the azide ion. These presumably involve the partial unfolding of the protein chain in such a way that one of the six necessary iron co-ordination positions becomes inaccessible. Banaschak and Jung have also made the remarkable observation that on longer exposure to urea the methaemoglobin forms a second product, spectroscopically identical with the first, which, however, on dithionite reduction forms a haemochromogen, having the characteristic spectrum and behaviour of such a substance. Tsushima and Kawai (1956), using sodium dodecyl sulphate as a denaturant, likewise find that methaemoglobin gives rise successively to two products having identical absorption spectra in the visible, and separable electrophoretically. These are said to represent discrete stages in the unfolding of the polypeptide chains, resulting in the exposure of nitrogen-containing groups, capable of co-ordinating with haem iron to give a hemichromogen. It would be expected that in cases of mild denaturation, electrophoretic mobilities would be altered by elimination or exposure of charged groups or by changes in their *p*K values. This compound will enter into an equilibrium with the cyanide ion, in which either the latter or co-ordinated globin is displaced, according to experimental conditions (Tsushima, 1956; Scheler, Jung, and Hoffmann, 1954).

White and Kerr (1957) have studied spectrophotometrically the denaturation of adult and foetal haemoglobins by nicotinamide, guanidine, sodium benzoate, and sodium salicylate as well as urea. The absorption curves obtained before

*Saha (1956) working with chicken haemoglobin reports differences in absorption band positions between the native oxyhaemoglobin, and the dialysed and undialysed alkali-resistant residues.

and after dithionite reduction are different from those arising from alkali denaturation at *p*H 9–11 and are ascribed to denaturation intermediates.

It is obviously worth pursuing any indications of the appearance of modified forms of haemoglobin elsewhere, bearing in mind the implications that such manifestations might have when related to the separation and characterization of components in haemoglobin mixtures. The existence of slightly modified forms of haemoglobin, which have all the significant properties associated with the native protein, is further confirmed by the products of recombination of isolated haem and globin. That the reconstitution of an oxygen-carrying pigment from heat-coagulated or acid-denatured haemoglobin is often possible has long been known (Mirsky and Anson, 1930; Holden, 1937), though such substances evince marked differences from haemoglobin in its native state.

Okken (1954) found that his preparation of recombined haemoglobin exhibited heterogeneity in free boundary electrophoresis, the fast and slow components being present in the ratio of 3:1. Other preparations of reconstituted haemoglobin have been carried out. Rossi-Fanelli and Antonini (1958), for instance, found that their samples of reconstituted haemoglobin gave oxygen dissociation curves identical with those of the native protein. Rossi-Fanelli, Antonini, and Caputo (1958) describe a reconstituted haemoglobin, which is said to have the physical, chemical, and physiological characteristics of the native substance, and is derived from an electrophoretically pure globin. On the other hand, Kistler, Buri, and Nitschmann (1953) have prepared a similar pigment from globin, which, though electrophoretically pure, is shown in fact to be heterogeneous by the nature of its solubility curve. The work of Jope, Jope, and O'Brien (1949) on the properties of recombined haemoglobin casts especial light on the existence of denaturation intermediates. These authors find that globin as usually prepared from Hb-A has a tryptophan fine-structure band in the 289.0–290.3 mμ region compared with 291.0 mμ in Hb-A. This globin can be fractionated by salting out, when the fraction having the tryptophan band at the longest wavelength is the least prone to precipitation. All globin with a tryptophan band of wavelength shorter than 290.6 mμ was rejected, and the resulting material, when recombined with haem, gave a haemoglobin having the band at 291.0 mμ. It was stated that the globins having the tryptophan band at a shorter wavelength were none the less in the native state by all reasonable criteria. Jope *et al.* also list the wavelengths of the Soret peak of the various preparations, and it is seen that all of them, with the possible exception of the carboxy-derivative of the sample prepared as above, have bands at slightly shorter wavelength than Hb-A. The corresponding methaemoglobins all have a Soret peak in the identical position with methaemoglobin A. Araya, Nakanishi, Nakajima, and Kaminaga (1955) have also prepared a reconstituted methaemoglobin directly by the recombination of haemin and globin at *p*H 7. This has a somewhat changed spectrum in the Soret region, although the protein bands are not significantly altered. It is clear then, considering also the electrophoretic heterogeneity of Okken's samples, that modified haemoglobins exist which are to be considered as undenatured but nevertheless behave as separate species. It is an interesting speculation whether such variants might occur pathologically.

An example of the occurrence of a modified haemoglobin is that described by Cook and Morrison (1956); ion exchange chromatography of cord blood haemoglobin gives, according to these authors, at least three fractions, the fastest (about 70%) being Hb-F, the slowest (about 10%) running in the same position as A_2 of normal adult haemoglobin, and the remainder corresponding to Hb-A. The portion of the cord blood haemoglobin remaining undenatured at *p*H 12.7, having the spectroscopic features of oxyhaemoglobin and remaining unprecipitated by one-third saturated ammonium sulphate solution, was also chromatographed. The extraordinary result emerged that only the slowest zone had completely resisted denaturation, and that the residual Hb-F was eluted more slowly from the column after treatment. Apart from this unexpected behaviour of the slow component, it seems clear that the Hb-F, though ostensibly still native haemoglobin, has been modified and behaves as a new species. Methaemoglobin is not likely to be involved here, since the spectrum is said to be characteristic of the oxy-pigment. It is also a matter of common experience (Morrison and Cook, 1955b; Boardman and Partridge, 1955, etc.) that methaemoglobin separates on ion exchange columns as a well-defined brown zone, more strongly adsorbed than haemoglobins.

The question arises then whether haemoglobin fractions isolated by chromatography and electrophoresis have been sufficiently scrutinized spectroscopically. The modified Hb-F, for

instance, of Morrison and Cook is to have the spectral characteristics of oxyhaemoglobin, but presumably neither these nor other authors in the field have examined their samples sufficiently carefully to show up small spectral differences of the kind observed by Jope and the present authors. It is proposed to show that such modifications of the haemoglobin molecule, which would not be observed by a cursory spectroscopic examination, could be brought about by far less drastic means than those so far mentioned.

In the first place sufficiently strong adsorption on columns (Kruh, 1952; Boardman and Partridge, 1953; Cook and Morrison, 1956, etc.) or supporting media in zone electrophoresis could result in damage to the protein in the same way as is known to happen in adsorption on alumina (Prins and Huisman, 1956a). This possibility could be resolved by rechromatography of individual zones, as carried out by Boardman and Partridge (1955) and by Morrison and Cook (1955b), though in the latter case the shape of the elution curve of the rechromatographed sample is equivocal. Itano *et al.* (1956) suggest that the poor resolution of haemoglobins on paper at *p*H values of 7 and below is due to denaturation, presumably by adsorption or conceivably by heat. Modiano (1957), in his interesting paper on the separation and estimation of minor components in Hb-A, suggests that the A_3 fraction observed by him on paper (though not resolved by Kunkel *et al.* (1957; Kunkel and Wallenius, 1955) on starch) may be an adsorption phenomenon of this kind.

More particularly, modification of some of the haemoglobin might well result from the haemolysis procedure applied to the initial preparation of the sample from the erythrocytes. Huisman and Schreuder (1957) have examined and compared a number of methods of haemolysis, and have found that none of them leads to any denaturation of the protein (but see also Baar and Hickman (1941) for the effect of saponin). On the other hand, they give no indication of having examined the spectra of their haemolysates sufficiently closely to show up small differences of the kind mentioned.* It has long been known, for example, that freezing can have a deleterious effect on proteins (Nord, 1936; Oncley, Gurd, and Melin, 1950), which is a fact to be borne in mind where freezing and thawing are used to effect haemolysis; indeed, Berry and Chanutin (1958) found that haemolysates thus prepared gave rise to abnormal patterns in free boundary electrophoresis, a large number of components being obtained. It may be mentioned also that Hb-H is said to be destroyed completely on freezing (Rigas, Koler, and Osgood, 1956). Huisman and Schreuder conclude that the best method of haemolysis is to add toluene and this appears in any case to be the most widely applied technique. Now Betke *et al.* (1956c) have shown that even a brief homogenization of toluene with oxyhaemoglobin solutions produces extensive denaturation, with formation of methaemoglobin in the undenatured portion. It would not therefore be surprising if intermediate species or denaturation products were to be formed, which could give rise to severe artefacts in fractionation experiments. Berry and Chanutin (1958) have in fact reported unsatisfactory results with haemolysates prepared by Drabkin's (1946) procedure. Prolonged exposure to air (Tsen and Tappel, 1958), as well as adsorption on vessel walls and mechanical agitation (Wu and Ling, 1927) or frothing, during, for instance, carboxylation (Nakanishi, Nakajima, Kaminaga, and Araya, 1955), could also be responsible. Before proceeding to examples of observations which suggest an interpretation in these terms, it should also be mentioned that effects of heterogeneity can apparently arise from intermediate oxidation stages between haemoglobin and methaemoglobin. Itano and Robinson (1956) observed the appearance in free boundary electrophoresis of components in partially oxidized carboxyhaemoglobin, which they believe to represent intermediate stages in the oxidation to methaemoglobin, the haem groups being successively affected. Itano and Robinson have more recently (1958) reported the appearance of intermediates in the conversion of methaemoglobin into its cyanide derivative. (This work as it stands is rendered ambiguous, however, by the observed fact (Remmer and Meyer-Wilmes, 1958) that the reaction of oxyhaemoglobin with ferricyanide is far from stoichiometric: according to the results of Remmer and Meyer-Wilmes, the proportions of reagents used by Itano and Robinson should not in fact give the ratios of components that they obtain electrophoretically. It is said (Itano and Robinson, 1958) that if oxidation is carried out at *p*H values below about 7, no sulphydryl groups are oxidized. This, however, does not guarantee a stoichiometric reaction with ferricyanide, and Steinhardt (N.A.S.-N.R.C. Conference, 1958,

*Itano *et al.* (1956) note that trailing and poor separation in paper electrophoresis may be ascribable to effects arising from the preparation of the haemoglobin solution and it is known that such effects also increase rapidly with the age of the sample (Motulsky *et al.*, 1954).

p. 154) has commented that no ferrocyanide ions could ever be separated from methaemoglobin, no matter how long it was dialysed. Moreover, the authors have noted that the ultra-violet protein band minimum in methaemoglobin is partially filled in, even when the oxidation has been carried out at a *p*H value below 7.

Morrison and Cook (1957) claim to have separated some of these intermediates by ion exchange chromatography, and Huisman *et al.* (1958) have obtained a number of components by applying solutions of haemoglobin, which have been exposed to the action of small amounts of ferricyanide, to modified cellulose columns. Spectra of the fractions are given, but the extinction values of the methaemoglobin and oxyhaemoglobin maxima are not consistent with an explanation in terms of stepwise oxidation of the haems.* Cases where such intermediates might pass as separate haemoglobin species are not slow to suggest themselves, particularly where the oxidation process has been arrested, for instance by the transference of a sample to a cold room. Such intermediates might also be anticipated in the blood of subjects with methaemoglobinaemia. Horecker (1943) found that after standing about two weeks haemoglobin solutions develop a band at 820 mμ, suggesting the formation of methaemoglobin, but no corresponding decrease in CO-combining capacity is observed.

Modification of haemoglobin of the type described would lead to changes in some of the chemical and physical properties of the molecule. The effect of slightly elevated temperature and unfavourable conditions of *p*H and ionic strength on the number of titratable sulphydryl groups (Benesch *et al.*, 1955; Murayama, 1957a, 1958) and the nature of the oxygen dissociation curve (Takashima, 1954, 1955; Wyman, 1948, etc.) requires no further elaboration here. Kubo (1956) has shown that somewhat denatured haemoglobin, still capable of oxygen binding, has an equilibrium curve, the shape of which is such as to suggest that one only of the four haems is affected. Such deviation from the behaviour of fresh haemolysates is exhibited by solutions which have merely been stored in the cold for a matter of a few days. Betke's experience (1957; Betke *et al.*, 1956b) that the rate of oxidation of oxyhaemoglobin with sodium nitrite depends on the means of preparation of the solution is explicable in terms of modification of the molecule during haemolysis or subsequent treatment. It is likely also that such results could arise from a partial oxidation. The ease with which sulphydryl groups are oxidized in haemoglobin, not only by such reagents as the ferricyanide ion (Remmer and Meyer-Wilmes, 1958) but also by atmospheric oxygen (Tsen and Tappel, 1958) is well known. Taylor (1955) has shown that the oxidation of canine haemoglobin, which is attended by the removal of two active sulphydryl groups, gives rise to a haemoglobin having completely new physical properties, in particular increased solubility, and a changed electrophoretic mobility. The bearing that this observation could have on the minor components of normal adult haemoglobin is self-evident. Other examples of the same kind spring to mind, but the important question of the minor components of normal adult haemoglobin is of specific interest.

Minor fractions of normal adult haemoglobin were obtained by Morrison and Cook (1955b) from oxyhaemoglobin by column chromatography on ion exchange resin at *p*H 6.3. The minor fractions constituted 10% and 6% respectively of the total and were designated A_3 and A_2. Rechromatography of the main peak gave no distinct heterogeneity, but the shape of the elution curve as reproduced (Morrison and Cook, 1955b, 1957) suggests that small quantities of A_3 might have reappeared. Subsequently, moreover, Morrison (N.A.S.-N.R.C. Conference, 1958, p. 167) has reported that under suitable conditions the first fraction could indeed be made to yield an extra component, which he surmises to be methaemoglobin reductase.* Three minor haemoglobin components, all fast running, were obtained on columns by Allen *et al.* (1958), but no slow component (A_2) was found. Again, Huisman *et al.* (1958) on modified cellulose columns find a fast-running component of about 10%, another of 0.5 to 2%, associated, however, with a wave of increased *p*H and therefore presumably spurious, and a slow component of 0.5 to 2%. (It is significant that the same authors found no increase in the proportion of slow-running component in thalassaemia major haemoglobin.) The chromatography of cord blood haemoglobins by Cook and Morrison (1956) has already been mentioned, and it must be said that the concentration of the minor component, viz., 10%, accompanied by 20% of normal Hb-A, seems too high to be acceptable. No component

*Attention should be drawn to the complex nature of the interactions occurring in mixtures of oxy- and methaemoglobin discussed by Scheler and Jung (1954), and said by them to involve the possible oxidation of sulphydryl groups in the globin.

*The A_3 component has been studied by Kunkel and Bearn (1957) by incorporation of Fe^{59}, and appears to be a first natural degradation product of Hb-A.

of this sort has been reported by other authors, and has not been observed by the present authors on any of a large number of ion exchange columns under vrations conditions. Such minor components as have been reported in association with Hb-F (Prins and Huisman, 1956b; Allen *et al.*, 1958) have been fast-running ones. The abnormally high stability of Morrison and Cook's component in alkali makes it highly probable that this material is either a modified or bound pigment.

Although minor components have been observed and estimated by paper electrophoresis (see above) the only other preparative separation has been by Kunkel and his co-workers of A_2, using starch slab electrophoresis (Kunkel and Wallenius, 1955; Kunkel *et al.*, 1957). The A_2 was distinguished only as a pointed front of the main zone, suggesting strongly that this component, if not a natural degradation intermediate (Kunkel and Bearn, 1957), is a denaturation product, resulting from adsorption effects, etc., or the haemolysis procedure. Whether the A_2 component of Morrison and Cook is identical with that of Kunkel *et al*, or indeed the components claimed by Van Fossan (1954), Roche and Derrien (1951), Roche *et al.* (1952), and other workers by various methods, is not known, and here again precise measurements of the absorption spectra, particularly in the Soret region, might give an indication as to the nature of the product. Kunkel *et al.* (1957) are uncertain about the homogeneity or otherwise of their A_2 sample because of the rapid denaturation which sets in even at 4° C. Electrophoresis of the A_2 in a supporting medium of starch block or polyvinyl chloride shows a slower component, but this may be a derivative of A_2, presumably a product of partial denaturation. It is by no means unlikely that the minute A_2 and "A_4" components separated by Masri *et al.* (1958) and Ceppellini *et al.* (1958) on starch from enormous haemoglobin samples are products of this kind.

Hoch (1950) found by free boundary electrophoresis that fresh haemoglobin solutions contain two small fast components. If a solution was kept for two weeks a small new peak appeared between the main peak and the slower minor component. The situation was further complicated by the work of Berry and Chanutin (1957, 1958), who were concerned with the storage of blood. These authors found that haemolysates of very fresh blood, when examined in a free boundary apparatus in 0.05 M cacodylate buffer at *p*H 6.5, gave two components, the minor one comprising 25% of the total red cell extract. On standing, the proportion of this component gradually decreased, its durability varying between samples from healthy and anaemic subjects. This observation is clearly not compatible with the results of other authors mentioned above. What it does suggest, however, is that the minor component is due to a reversible modification of the Hb-A or to binding with erythrocyte constituents. That variations are due to aberrations in the number of haems per protein entity cannot be ruled out, though Beutler (1958) has shown that such molecules are not in fact synthesized, even under conditions of extreme iron deficiency.

One further debatable question must be discussed: the nature of the so-called haemoglobin M. It must first be said that the denaturation of methaemoglobin with mild reagents, such as sodium salicylate, urea and urethanes, has been carefully studied (Schlegel and Johnson, 1949; Banaschak and Jung, 1956) and the nature of the irregular denaturation curve has led Chalopin (1953) to postulate the heterogeneity of methaemoglobin. Steinhardt and Zaiser (1955), on the contrary, believe that this behaviour arises from steps in the denaturation process,* and Derrien and Reynaud (1955) find that electrophoretic heterogeneity in solutions containing Hb-F disappears on oxidation. Chalopin (1958) has observed that sodium salicylate denatures methaemoglobin without precipitation, if the concentration of the denaturing agent is high. Schlegel and Johnson (1949) have followed the process spectrophotometrically and have also found that it is not accompanied by a volume change.

Since this is usually considered one of the characteristics of the denaturation of proteins, the methaemoglobin should be regarded as "modified" rather than denatured. Chalopin (1958) moreover gives reasons for supposing that a wide range of products, in various stages of attack, is present. It is likely in any case that the reduction products of such a methaemoglobin would be scarcely distinguishable from native haemoglobin. Banaschak and Jung (1956) and Schlegel and Johnson (1949) give absorption spectra in the visible region of intermediate stages in the reaction of methaemoglobin with sodium salicylate

*The nature of the reversible and irreversible stages in denaturation is as yet obscure. The early stages appear to involve an unfolding or swelling of the peptide chains, followed by the breakage of S-S bridges and possibly their subsequent regeneration in different positions (Stauff, 1956). It seems possible that ultraviolet spectrophotometric examination of the globin bands at various stages in the denaturation process may furnish information (Nakajima, Nakanishi, Kaminaga, Araya, and Kikuchi, 1955).

and urethanes, and some of these bear a marked resemblance to the visible spectra of methaemoglobin M shown by Hörlein and Weber (1948), Kiese *et al.* (1956), and Heck and Wolf (1958). Both the latter groups of authors have found that the spectroscopic deviations do not extend to any of the haemoglobin M derivatives, and that the abnormality lies in the globin and not in the haem. This is interesting, but does not exclude the possibility that the abnormal methaemoglobin is due not to a genetic variant of the haemoglobin but merely to a modification of the protein by erythrocyte constituents. This could occur by attack on the haemoglobin with methaemoglobin formation in the first or a subsequent stage. The latter is evidently the more likely alternative, since Kiese *et al.* find that the rate of oxidation of haemoglobin of a subject suffering from this form of methaemoglobinaemia is almost twice as great as that of normal adult haemoglobin (with consequent overloading of the methaemoglobin reductase system in the erythrocyte). Also Gerald *et al.* (1957) claim to have isolated by starch block electrophoresis a precursor haemoglobin, which when oxidized gives the spectrum of the abnormal methaemoglobin M. The resolution is not good, however, and the results are vitiated by contamination of the haemoglobin with the incompletely separated methaemoglobin. (A similar separation has been reported on paper by Pisciotta, Ebbe, and Hinz (1957), who find a slow-running haemoglobin to be present. Their sample contained 11% of alkali-resistant pigment, but no mention is made of the appearance of Hb-F on paper. The authors conclude that the slow-running material, Hb-M, is incapable of carrying oxygen. The argument, however, is somewhat obscure and in any case runs counter to the results of Kiese *et al.* (1956), who find that the oxygen dissociation behaviour of their specimen is normal.) More recently Gerald (1958) has described in detail the spectroscopic and electrophoretic characteristics of an Hb-M specimen. It was found in particular that a clear separation on starch slab could be obtained of two components in the haemolysate after ferricyanide oxidation. One of these exhibited all the normal characteristics of methaemoglobin, but the other showed an extraordinary anomalous reaction to sodium cyanide. On treatment with small amounts of this reagent a double Soret peak was observed, and this was eliminated, with simultaneous formation of the characteristic cyanmethaemoglobin spectrum, only on addition of a large excess of cyanide ion. This suggests that an unusual type of linkage is present in at least one of the haem groups of the molecule, in which a bound component is displaced only with difficulty by cyanide. Such an effect could arise from an extra link to the globin, arising from partial denaturation or unfolding of the protein, or to an extraneous substance. This situation is strikingly reminiscent of the supposed equilibrium between bonded globin and cyanide ion described by Tsushima (1956).

It would be of interest in connexion with the Hb-M problem in general to seek a pathological denaturing agency within the erythrocyte.

[*The second part, together with a list of references, will appear in the March issue.*]

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RENAL FUNCTION IN BURNED PATIENTS AND ITS RELATIONSHIP TO MORPHOLOGICAL CHANGES

BY

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Renal dysfunction not infrequently follows severe burns, but much of its nature and pathogenesis is as obscure as its relationship to mortality. Previous studies showed that the blood urea rose considerably both in subjects who became severely oliguric, and in others with a normal urine flow, whilst the kidneys of both groups of patients often portrayed the same histological picture of diffuse distal tubular necrosis (Sevitt, 1956a). The high plasma : urine (P : U) ratios of sodium and chloride found indicated renal-tubular ability to absorb these ions in spite of the tubular necrosis and azotaemia. These findings did not support the thesis that acute renal failure is always characterized by anuria or severe oliguria and that degeneration or necrosis of the renal tubules is its morphological expression or pathological basis.

The present investigation of renal function was planned to take into account the rapid changes in blood volume, haemodynamics, and fluid exchange which occur in extensively burned treated subjects and to time the onset of changes in renal function. Renal function is influenced by the early onset of oligaemia, mainly from the exudation of a plasma-like fluid into the burned area, by the administration of fluid which combats the oligaemia, by haemoglobinuria from red cell destruction, and by cardiovascular and hormonal changes. During the first two or three days fluid continues to be lost into the burn oedema ; then the oedema is reabsorbed and an additional load is placed on the kidneys. Serial hourly specimens of urine with appropriate blood samples were therefore examined to establish the patterns of glomerular and tubular function, and these were related to the histological changes in the kidneys of those who subsequently died.

The results indicate that an important defect in acute renal failure is glomerular even though tubular necrosis and blockage by casts is often present. A sufficient fall in the glomerular filtration rate (G.F.R.) precipitates azotaemia, but the presence or absence of oliguria is determined both by the degree of reduction in filtration and by the tubular reabsorption of water. A decrease in the latter may adequately counterbalance the reduced G.F.R. and the urine flow may then be normal or even polyuric. Oliguria results when the decreased reabsorption does not balance the severe reduction in filtration. Continued tubular activity is indicated by the low urinary concentration of sodium and chloride, by the wave of increased potassium excretion after burning, and by the renal ability to discriminate in the handling of water, creatinine, urea, sodium, potassium, and chloride. Similar patterns of electrolyte excretion were found in non-azotaemic, temporarily azotaemic, and persistently azotaemic subjects with or without oliguria and with or without tubular necrosis. The relationship between severe azotaemia and tubular necrosis is inconstant and the major functional defect is glomerular and not tubular in origin.

PATIENTS AND METHODS

Patients

Table I gives the necessary clinical details of the patients studied.

Seventeen patients (14 women) were studied, eight of whom were 12 years of age or younger, six between 36 and 54 years of age, and the remainder older than 65 years. In four patients the extent of burning was 18% to 30% of the body area, 30% to 40% in another four, and 40% to 90% in the remaining nine. Eleven patients died three to 18 days after burning. Four of the six survivors had burns between 20% and 30% of the body area.

Thirteen patients (Cases 4 to 7 and 9 to 17) were extensively burned (25% to 90%) "shock cases" admitted consecutively. Cases 1 to 3 were added as examples of less extensively burned subjects (18% to 20%). Case 8 was admitted from another hospital two days after burning ; the others were admitted between one and three and a half hours after the accident.

TABLE I
THE PATIENTS

Case No.	Sex and Age (Yr.)	Percentage Body Area Burned	Intravenous Therapy: Period from Burn to Onset of Therapy (hr.)	Intravenous Therapy: Litres of Plasma and Blood in First 48 Hours	Lived or Died (Survival Period in Days)	Clinical and Necropsy Notes
1	F 74	18	2	3·7	D, (3)	Died rather suddenly. Atelectasis lower lobe left lung. Nephrosclerosis
2	F 10	20	1½	5·4	L	Primary excision and grafting
3	F 40	20	2	8·2	L	
4	M 12	25	1	8·2	L	Primary excision and grafting
5	F 53	30	2	14·3	D, (5)	Primary excision and grafting. Considerable post-operative bleeding into dressing. Nephrosclerosis
6	F 3½	30	1	1·2	L	
7	F 71	35	2	4·8	D, (8)	
8	F 9	36	*	3·4	D, (18)	Acute haemolytic jaundice. Bronchopneumonia. Small acute duodenal ulcer. Slight fatty liver
9	F 12	40	3½	11·1	L	
10	F 66	40	1½	4·8	D, (6)	Flabby, fatty heart. Acute duodenal ulcer. ? Septicaemia
11	F 45	45	3	11·0	D, (12)	Hyperpyrexial attacks
12	F 5	50	1	5·4	L	Primary excision and grafting
13	F 54	50	2	5·9	D, (9)	Bronchopneumonia. Focal nephrosclerosis
14	F 36	60	2½	14·2	D, (5)	Bronchopneumonia. Moderately fatty liver
15	F 2	65	2½	4·8	D, (11)	Lobar atelectasis. Moderately fatty liver
16	M 45	85	3	11·3	D, (4)	Died rather suddenly. Inhalation of vomit
17	M 5	90	1	5·9	D, (5)	Burn of larynx, some respiratory obstruction. Necropsy—acute pulmonary emphysema, cerebral swelling, multiple tiny haemorrhagic erosions in gastric mucosa, haemorrhagic ileum

* Patient treated in another hospital during first two days.

Continuous intravenous therapy for about 48 hours consisting of reconstituted dried citrated plasma supplemented by stored blood and oral fluids was directed against oligaemia and haemoconcentration ; it was controlled by repeated haematocrit determinations, by observations on the urine flow, and by close clinical examination. During the first two or three days daily estimations of the red cell volume by the P^{32} and Cr^{51} techniques were made by Dr. E. Topley and her colleagues (see Topley and Jackson, 1957) and guided the transfusion of blood.

In Cases 2, 4, 5, and 12 primary excision and grafting of much of the burn was performed seven to 18 hours after burning. The considerable operative and post-operative blood loss was replaced, guided by swab weighing and red cell volume determinations, but one patient (Case 5) went through a post-operative oligaemia lasting several hours before it was corrected by transfusion.

The burns of the other patients were dusted with lactose-penicillin powder and covered with sterile towels during the shock period. Grafting operations were done two to three weeks after burning.

After the shock period a high-protein, high-calorie diet was given by intragastric drip until a normal diet was judged appropriate. The diet was altered to a high-calorie, low-protein kind in those with severe oliguria.

Methods

Frequent blood sampling, accurate haematocrit estimation, and red cell volume studies were facilitated in nine patients (Cases 6, 7, 9, 10, 11, 12, 14, 15, and 17) by guiding a polythene catheter into a large central vein under local anaesthesia soon after admission. Otherwise blood was obtained by venous punctures.

Plasma creatinine was repeatedly determined in 12 patients (Cases 4, 6, 7, 9 to 17) three to seven times in the first 24 hours after burning, two to four times in the second 24 hours, one to four times in the third period, and then daily ; occasional estimates were made in others. In two patients (Cases 13 and 16) insufficient blood necessitated the pooling of several hourly specimens and the creatinine values were plotted at the midpoint of the period. Plasma or blood urea was repeatedly estimated in 15 patients ; in 10 (Cases 4, 5, 7, 9 to 12, 14 to 16) it was determined two to four times in the first 24 hours, one to four times in the second and third 24 hours, and then daily. Plasma sodium and potassium were estimated as often or more frequently in the first 24 hours, but plasma chloride and bicarbonate were determined daily or less often. The serial plasma creatinine, urea, and sodium values were plotted against time on semilog paper and values were extrapolated for clearance and other calculations.

An indwelling catheter was passed on admission and the bladder was emptied. Urine was collected hourly during the first 48 hours and then at four-hourly or longer intervals, but in six patients (Cases 4 and 12 to 16) hourly collections were continued for 60 to 100 hours. In 13 patients serial estimations of the creatinine, sodium, and potassium were made. Repeated urea analyses were made in seven patients (Cases 6, 7, 9, 10, 12, 14, and 15) and there were daily estimates in six others. Serial urea, inulin, and

TABLE II
SUMMARY OF FINDINGS

Case No.	Haemoglobinuria	Pattern of Fall in G.F.R.	Azotaemia: Type	Azotaemia: Highest Plasma Urea (mg. per 100 ml.)	Azotaemia: Highest Plasma Creatinine (mg. per 100 ml.)	Oliguria	Fraction of Filtered H_2O Excreted in Urine (Type)	Fraction of Filtered Na Excreted in Urine (Type)	Terminal Concentration in Urine (mEq./litre): Na	K	Cl	Renal Histology: Tubular Necrosis	Hydropic Change in Proximal Tubules	Hyaline Droplet Change in Origin of Proximal Tubules	Fatty Droplet Change in Glomerular Tufts
1	0		0	57	—	0			34	106	22	None	0	0	+
2	0		0	37	—	0									
3			Temporary	112	1·7	0									
4	+	Type A	0	46	1·2	0	A	B							
5	++		Severe, persistent	230	—	Severe			77	28	21	Focal D.T.N.	0	0	+
6	0	,, A	0	—	0·8	0	A	A							
7	++	,, B1	Severe, persistent	496	15·1	Severe	B	B	20	55		Necropsy refused			
8	++		,,	179	3·5	0						Diffuse D.T.N.	0	++	0
9	0	,, A	Temporary	102	2·2	0	A	B							
10	++	,, B1	Severe, persistent	292	6·2	0	B	A	9	29		Diffuse D.T.N.	+	0	+
11	++	,, B1	,,	250	5·2	0	B	A	11	32	7	P.T.N. ++	0	0	++
12		,, A	Temporary	80	1·1	0	A	B							
13	0	,, B2	Severe, persistent	184	2·3	0	B	A	10	70		None	0	+	++
14	++	,, B2	,,	273	4·9	0	B	B	10	64		P.T.N. ++ Focal D.T.N.	0	0	+++
15	+	,, A	Temporary	113	4·5	0	A	A	6	44		"Healing" focal D.T.N.	0	0	+
16	0	,, B2	Severe, persistent	—	4·7	0	B	A	5	53		None	+++	+	+++
17	++	,, B1	,,	229	7·4	Severe*	A	A	56 (at 24 hr. after burning)	106		Diffuse D.T.N.	++	0	+++

Pattern of fall in G.F.R.: Type A = temporary fall with return to normal; Type B1 = early severe and persistent fall; Type B2 = early moderate fall with subsequent decline. *Fraction of filtered H_2O excreted:* A = early increase with return to normal; B = persistent and/or progressive increase. *Fraction of filtered Na excreted:* A = normal or decreased from admission. B = early increase with anuria* after 24 hours. D.T.N. = distal tubular necrosis. P.T.N. = proximal tubular necrosis. 0 = little or no oliguria.

creatinine clearances were calculated from the formula $\frac{UV}{P}$. Urinary chloride was determined in 11 patients, hourly in three (Cases 7, 12, and 14), and daily in the others.

Biochemistry.—Creatinine in plasma and urine was estimated by a modification of the method of Brod and Sirota (1948). The colour was read at 510 mμ at 60 minutes instead of 15 minutes after adding the alkaline picrate reagent.

Simultaneous inulin and creatinine clearances were carried out in Cases 12 and 14 for 12 and eight urine collection periods respectively during the shock phase, each period lasting one to two hours. For the inulin clearances a steady plasma concentration was attained by varying the concentration of the intravenous infusion.

Inulin was estimated by a modification of the method of Rolf, Surtshin, and White (1949). The water bath was at 70° C. instead of 75° C. and 15 g. instead of 18 g. of diphenylalanine per litre of acetic acid-HCl mixture was used. This raises the fructose/glucose differentiation from 1 in 50 to 1 in 92 (Graber and Hancock, 1955). The colour which developed was read at 520 mμ. All colorimetric readings were made on a "unicam" 600 spectrophotometer.

Plasma and urinary sodium and potassium were estimated by flame photometry (E.E.L.); plasma and urinary chloride by a modification of the method of Schales and Schales (1941) diluting with a nitric acid/sodium citrate buffer at *p*H 1.5 instead of water; and plasma or blood urea by a urease-Nesslerization colorimetric method. Urinary urea was determined by the alkaline-hypobromite technique. Lactose in urine was detected by the methylamine test (Fearon, 1946).

Histology.—Kidneys from 10 fatally burned patients were studied. The bodies were refrigerated soon after death. The interval before necropsy was less than eight hours in five cases (1, 8, 10, 15, and 17), in three others (Cases 5, 13, and 16) between 10 and 20 hours, and only in two cases was it greater than 24 hours (Case 11—27 hours; Case 14—48 hours). Blocks from both kidneys (2.5 to 3.5 cm. long) were fixed in neutral formol-saline (in Cases 5 and 14 additional blocks were also fixed in formol-alcohol).

Paraffin sections were stained by haematoxylin and eosin, picromallory, periodic-acid-Schiff, Gram, prussian blue, Pickworth's benzidine, and other methods. Frozen sections were stained for lipids by oil red O and counterstained by light green or aniline blue, the latter for outlining the glomerular basement membrane in relationship to glomerular lipid.

RESULTS

The results are set out in Table II.

Validity of Creatinine Clearance as a Measure of Glomerular Filtration Rate

We believe that variations in the published ratios of creatinine clearance to inulin clearance (Ccr : Cin) are mainly variations in the method of estimating plasma creatinine and are not differences in the tubular handling of creatinine. The present method for estimating plasma creatinine was used in more than 500 simultaneous creatinine and inulin clearances in a variety of cases and give a mean Ccr : Cin ratio of 0.87 (Graber, 1955). A similar mean value was obtained for the 20 clearance ratios made during the shock phase in two of the present cases (Table III).

TABLE III
SIMULTANEOUS INULIN AND CREATININE CLEARANCE VALUES (ML. PER MINUTE) IN TWO PATIENTS

Hours after Burning	Case 12		Case 14	
	Inulin Clearance (ml./min.)	Creatinine Clearance (ml./min.)	Inulin Clearance (ml./min.)	Creatinine Clearance ml./min.)
27	43	41		
28	53	43	42	40
29	—	—	40	40
30	37	26	42	38
31	32	24	39	31
32	38	33	27	20
33	48	41	52	42
34	18	18	—	—
35	18	18	24	19
36	25	28	41	45
46	28	26	—	—
47	16	17	—	—
48	22	22	—	—
Mean Ccr : Cin	0·90		0·89	

Case 12 (mean normal G.F.R. 35 ml. per minute) developed a slight temporary azotaemia and survived, whilst Case 14 (mean normal G.F.R. 90 ml. per minute) became considerably azotaemic and died with renal tubular necrosis. Note that the simultaneous values for inulin and creatinine clearances are very similar in each case, as are the mean ratios of Ccreat : Cin ratios.

It has been suggested that the low clearance $\left(\frac{UV}{P}\right)$ values of creatinine, inulin, urea, and other substances found in patients with tubular necrosis results from an unselective back diffusion into the blood of filtered water and solutes which the tubular damage permits (Phillips and Hamilton, 1948; Bull, Joekes, and Lowe, 1950). Azotaemia and the low creatinine clearance then results from back diffusion and not from a low G.F.R. The possibility of back diffusion cannot be eliminated, but if it occurs the affected nephron behaves like a capillary and not like renal tissue; the result is a reduction in the functioning nephron mass and not an alteration in function.

We offer three kinds of evidence that non-specific reabsorption is of little importance in determining urine composition. First, the Ccr : Cin ratio was identical in Cases 12 and 14 (Table III). One had severe azotaemia and tubular necrosis and the other rapidly recovered full renal function. Secondly, Curea : Ccr ratios varied considerably in the same patient ranging from 0.1 to 1.4 (Fig. 1). The ratios were generally low in the early period after burning, tended to continue low in severely azotaemic patients, but rose in the others. Low and varying ratios indicate tubular discrimination between urea and creatinine and do not support the theory of unselective back diffusion. Thirdly, the low sodium and

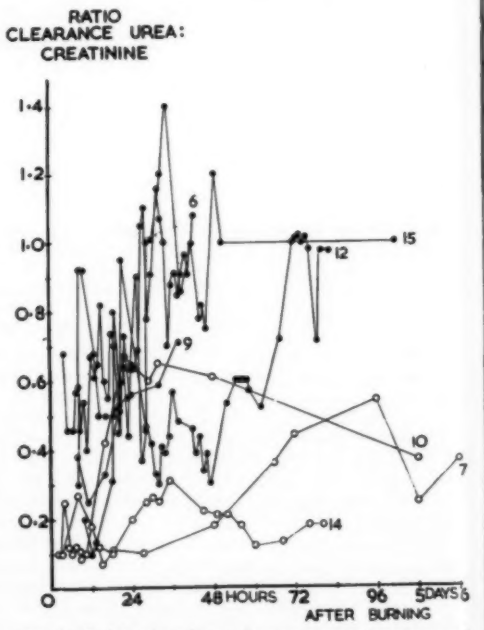

FIG. 1.—Serial ratios of urea clearance : creatinine clearance in seven patients at different times after burning. The black circles are the values from patients without azotaemia or with a moderate temporary azotaemia, whilst the open circles are from patients with severe persistent azotaemia. The numerals refer to case numbers. The ratios fluctuate and range widely from 0.1 to 1.4. Low ratios generally occur during the first 24 hours in all patients, but they tend to persist in those with severe azotaemia; this indicates differential tubular handling of creatinine and urea and does not support the theory of unselective tubular reabsorption.

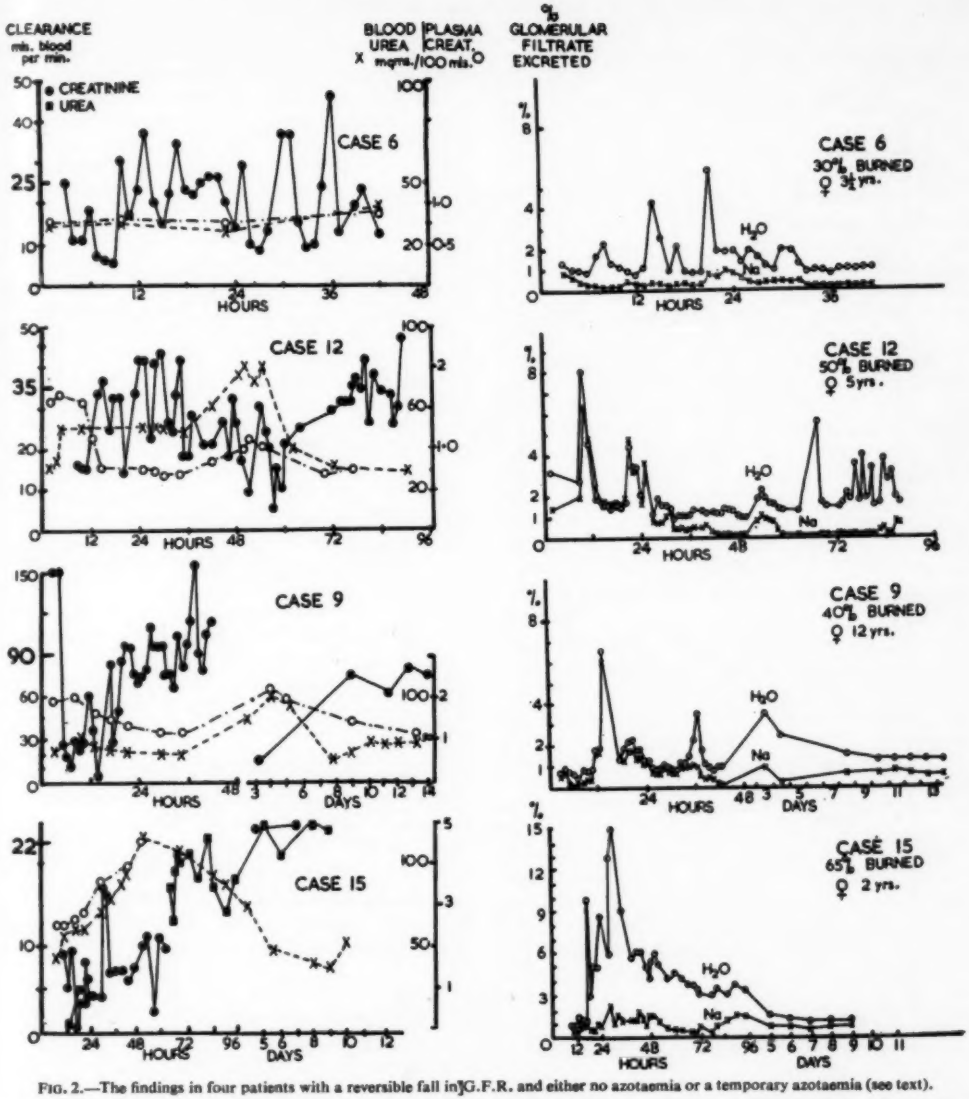

FIG. 2.—The findings in four patients with a reversible fall in G.F.R. and either no azotaemia or a temporary azotaemia (see text).

FIGS. 2, 3, and 4.—The graphs on the left show serial creatinine clearance (G.F.R.), urea clearance, plasma creatinine, and urea values. The expected normal G.F.R. (based on surface area derived from height and age) is shown in heavy type. The graphs on the right show the serial values for the percentage excretion of filtered water and sodium.

chloride concentrations (5 to 11 mEq. per litre) and high potassium levels in the urine before death in patients with tubular necrosis (Table II) are incompatible with the theory of unselective reabsorption. Such concentrations which are due to tubular activity could only occur if damaged nephrons were excluded from the urine flow.

Glomerular Filtration and Azotaemia

Within a few hours of beginning therapy the G.F.R. was found to be falling from a relatively normal or raised value (Cases 6, 7, 9, 10, 13, and 16) or was grossly reduced (Cases 11, 12, 14, 15, and 17, and Figs. 2, 3, and 4). During the first day or two some cases showed considerable

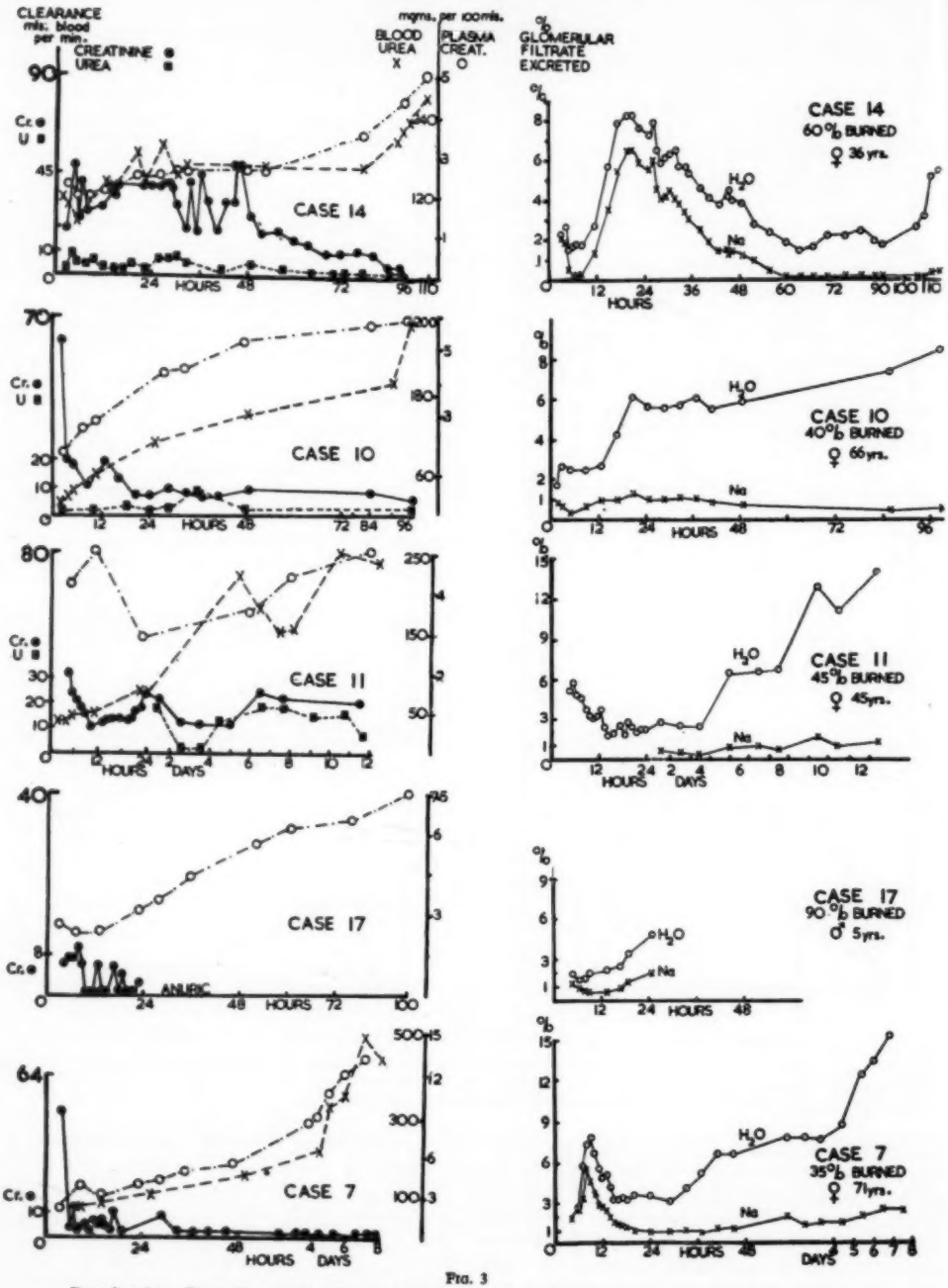

FIG. 3

FIGS. 3 and 4.—The findings in six patients with a persistent fall in the G.F.R. and a severe azotaemia (see text).

fluctuations in the G.F.R., often from hour to hour. Urine collection faults were excluded when the consecutive specimens of urine differed in creatinine, sodium, and potassium concentration as well as in volume. When similar concentrations were found, variations in creatinine clearance were considered to be collection faults and the results were averaged for the period. The rapidity of change points to the danger of drawing conclusions from only one or two spot estimates in any one case. Random and unrelated fluctuations in volume and specific gravity were also recorded by Didenko (1956) in consecutive three-hourly specimens of urine from extensively burned patients during the shock phase. Azotaemia developed when the fluctuations in the G.F.R. were below a mean value of about 50% of normal for 24 hours, but with higher mean values the periods of fall appeared to be too short to produce a significant effect, e.g., Case 6 (Fig. 2). The results may be divided into two main groups. Type A (Fig. 2) consists of patients with a considerable but temporary reduction in the mean G.F.R. either without azotaemia or coinciding with a phase of moderate azotaemia. Type B (Figs. 3 and 4) consists of patients with a persistently low G.F.R. and severe azotaemia, and may be divided into two subgroups, namely, B1, those in whom the G.F.R. fell to a low value soon after burning and remained low until death and in whom the azotaemia progressively increased; B2, those in whom the early fall in the G.F.R. was less considerable but in whom there was a further decrease and in whom the azotaemia developed in a variable fashion. One case had features of both subgroups.

A. Temporary Fall in G.F.R. with or without Reversible Azotaemia.—Eight patients (Cases 1 to 4, 6, 9, 12, and 15) fall into this category. Six were children between 2 and 12 years, and in the two adults the burns covered 20% of the body area or less. Serial creatinine clearances (Cases 4, 6, 9, 12, and 15) (Fig. 2) showed an irregular fall in the G.F.R. (lowest values 5% to 40% of normal), then an irregular return to normal—within 24 hours of burning in four patients and by 72 hours in Case 15. Cases 9 and 12 had a subsequent fall in the G.F.R. and an additional peak of azotaemia. The mean values ranged from 30% to 50% of normal during the first 12 hours in the different patients, from 18% to 90% during the second 12 hours, and from 45% to 110% of normal during the second 24 hours.

Four patients were without significant azotaemia (Cases 1, 2, 4, and 6), but in Cases 3, 9, 12, and 15 the blood urea and plasma creatinine levels rose for a few days, reaching maxima of 112, 102, 80, and 113 mg. % urea and 1.7, 2.2, 1.1, and 4.5 mg. % creatinine respectively (Table II) and then returned to normal. Six survived; Cases 1 and 15 died of causes unrelated to renal failure.

The urine output gave no cause for anxiety, the patients passing a relatively normal volume for their age.

B. Persistent Fall in G.F.R. and Severe Azotaemia.—Nine patients (seven adults) fall into this category (Cases 5, 7, 8, 10, 11, 13, 14, 16, and 17), all of whom died between four and 19 days after burning. The severe persistent azotaemia suggests that renal failure contributed to their deaths.

Cases 7, 10, 11, and 17 (Fig. 3) formed the subgroup B1 in whom the G.F.R. fell to between 8% and 17% of normal within a few hours of burning, and remained at very low levels until death supervened. The mean values during the first 12 hours varied from 10% to 25% of normal, during the second 12 hours from 10% to 15%, and during the second 24 hours from 7% to 25%.

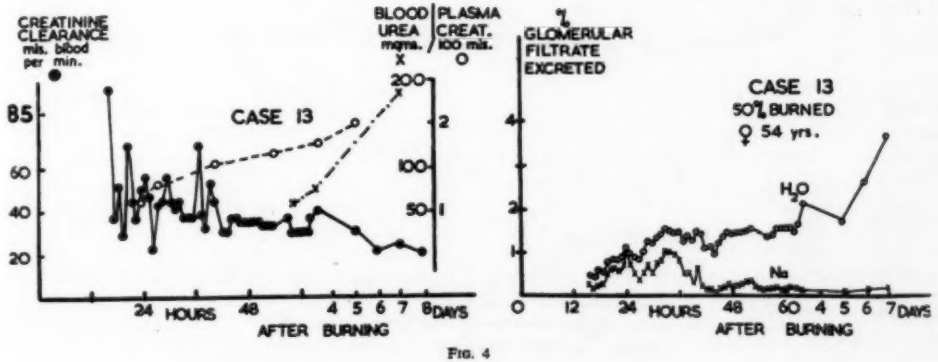

FIG. 4

Cases 13 (Fig. 4), 14 (Fig. 3), and 16 formed the subgroup B2 in whom the mean G.F.R. was 35% to 60% of normal during the first 24 hours and then declined to between 6% and 25% during the next few days. For example, in Case 14 the G.F.R. fell rapidly and fluctuated around a mean value of 40% of normal for the first 48 hours, then fell to 11% at 72 hours, and to 6% at 96 hours.

In Case 8 the G.F.R. on days three to five was about 10% of normal. Creatinine clearance studies were not made in Case 5, but urea clearances were only 0.1 to 0.2 ml. per minute.

The rise in the plasma creatinine and urea was considerable and generally progressive, but a temporary plateau or fall in azotaemia was found in a few patients. The maximum plasma urea levels ranged from 179 to 496 mg. per 100 ml. and the maximum creatinine values from 2.3 to 15.1 mg. per 100 ml. (Table II).

Cases 5, 7, and 17 had severe oliguria which developed within a few hours of burning, and the remainder had little or no reduction in urine flow. Case 7 passed only 279, 90, 116, 130, 210, 278, and 285 ml. of urine on successive days; Case 5 only 50 to 100 ml. per day, and Case 17 passed 70 ml. of urine in the first 24 hours and almost none after this.

In the others the urine flow was not greatly reduced except for short periods early in resuscitation and terminally, and was sometimes polyuric. For example, Case 10 passed 185 ml. during the first eight hours of resuscitation and successive 24-hour total volumes were 558, >700, >826, 812, and 730 ml. of urine. Nevertheless the plasma urea and creatinine mounted steadily from 32 mg. and 1.9 mg. respectively at two hours after burning to 292 mg. and 6.2 mg. per 100 ml. on the fourth day. Case 14 was somewhat different (Figs. 3 and 5). A period of urine flow averaging 33 ml. per hour from four to 11 hours after burning was followed by a polyuric output averaging 117 ml. per hour for the next 37 hours. The urine flow then decreased and this was associated with a sudden reappearance of numerous brown granular casts, and a further fall in the G.F.R., and a more rapid rise in azotaemia. The change suggested that a new "incident" had affected the kidneys.

Tubular Function

Our estimate of tubular function is based on the renal ability to reabsorb water, sodium, and

FIGS. 5 to 7.—Urinary output, serial urinary concentration of sodium, potassium, and chloride and blood urea values in three patients.

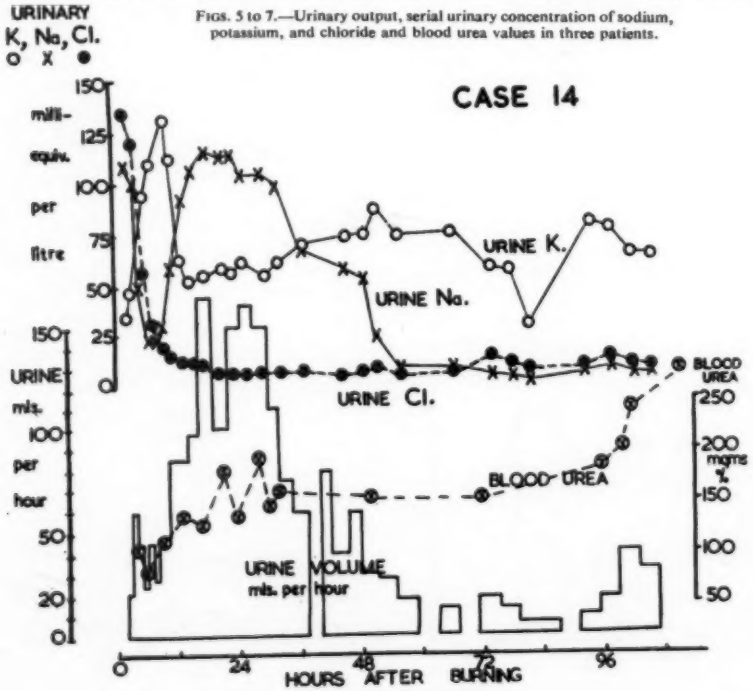

FIG. 5.—Case 14. Note the early onset of a low urinary concentration of chloride, the phases of low sodium excretion, the high urinary level of potassium, and the absence of oliguria in this patient who developed a severe, persistent azotaemia and extensive necrosis of the proximal renal tubules (cf. Case 12, Fig. 7).

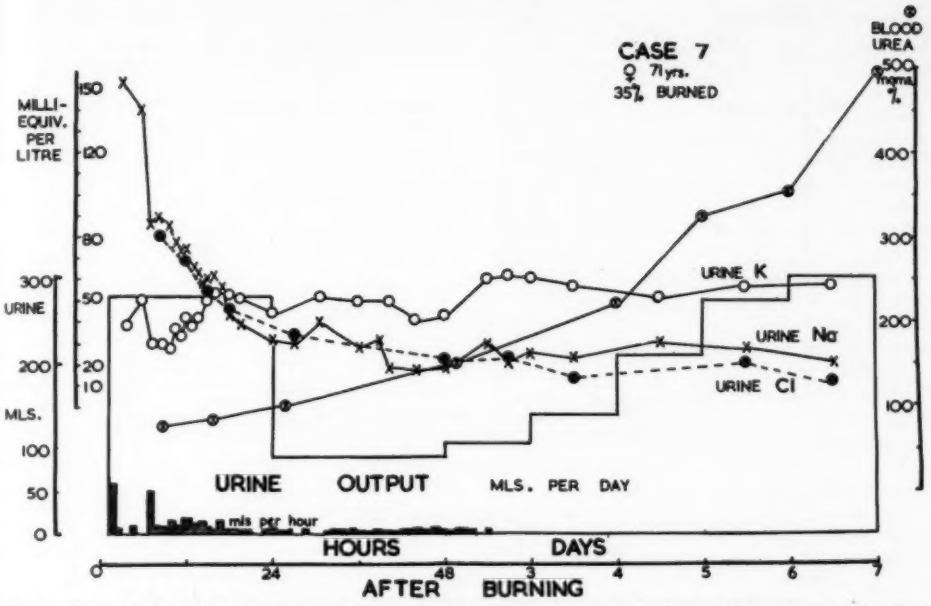

FIG. 6.—Case 7. In this severely oliguric progressively azotaemic patient there is a parallel development of low urinary concentrations of sodium and chloride and a moderately high level of potassium.

chloride, to excrete potassium, and to differentiate between these substances. The differential tubular handling of creatinine and urea has already been referred to.

Excretion of Electrolytes.—The general pattern of sodium excretion (Fig. 5) was a low urinary concentration sometimes preceded by a temporary high concentration. The chloride level rapidly fell to low values whilst the potassium level rapidly increased.

With the possible exception of one severely oliguric patient (Case 5) similar patterns of excretion of sodium, potassium, and chloride occurred in those with little or no azotaemia and in those with severe azotaemia.

Sodium and Potassium.—Three variations in sodium excretion could be distinguished.

(1) The urinary sodium concentration fell sharply after burning, low values were reached within 12 to 48 hours and were continued for days (Case 7, Fig. 6, Cases 10, 11, 15, 16, and probably Cases 1–3). For example, in Cases 15 and 16 urinary levels of only 10 to 15 mEq./litre developed by 24 hours after burning. The findings in Case 7, who developed severe oliguria, are of special interest: the high initial sodium concentration of 150 mEq./litre fell steadily to 30 mEq./litre at 24 hours, reached 17 mEq./litre at 40 hours, and thereafter remained between 20 and 25 mEq./litre until death on the eighth day. This indicates a tubular ability to reabsorb sodium even in the classical severely oliguric form of acute renal failure. Potassium excretion was usually higher than Case 7 indicates; for example, in Case 16 the concentration rose to 191 mEq./litre by 10 hours after burning and remained above 100 mEq./litre for 55 hours.

Case 17, who was anuric after 24 hours, may also belong to this group: the urinary sodium level fell from 113 to 56 mEq. per litre and the potassium level was high (100–110 mEq. per litre) before the urine flow ceased.

(2) One or two phases of a sodium diuresis lasting up to 24 or 48 hours preceded the fall to a very low sodium value whereas the potassium concentration was generally high. This pattern was found in six patients. In Case 12 (Fig. 7) the sodium concentration mounted to 180 mEq./litre; two episodes of a low concentration (10–20 mEq./litre) occurred, one between 40 and 50 hours after burning and the second between 60 and 96 hours (not shown in the figure). A potassium concentration of 110 mEq./litre developed within 12 hours. In Case 14 (Fig. 5) an initial

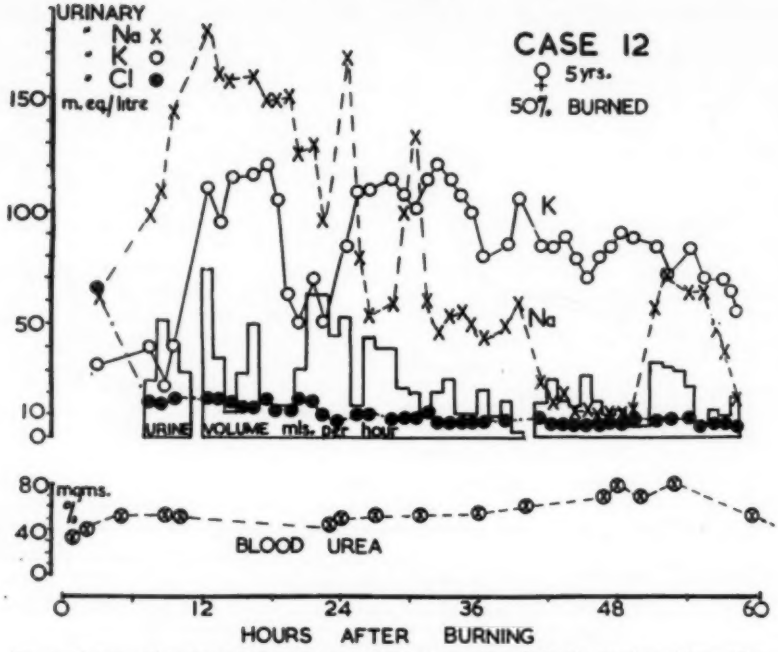

FIG. 7.—Case 12. Note the early onset of a low urinary concentration of chloride, the phase of low sodium excretion, the high urinary level of potassium, and the absence of oliguria (cf. Case 14, Fig. 5). This patient developed only slight temporary azotaemia and survived.

high sodium concentration (100–126 mEq./litre) and high to normal urine flow lasting two hours was followed by a reduced sodium concentration (20 mEq./litre) and urine flow lasting several hours; sodium and water diuresis then returned for 24 to 36 hours, after which oliguria and a low sodium level (5 to 10 mEq./litre) persisted until death on the fifth day. Potassium excretion increased to 125 mEq./litre within two hours of burning as the sodium level fell, and a few hours later fell as the sodium level rose.

(3) In one of the three severely oliguric patients (Case 5) low sodium and high potassium concentrations did not occur. Initially the sodium was 45 mEq./litre and then ranged from 50 to 96 mEq./litre and terminally was 77 mEq./litre on the fifth day. Potassium concentration slowly fell from 61 to 25 mEq./litre during the same period. The low P:U ratios for sodium (1.5 to 3) might be interpreted as evidence of impaired tubular activity, but the urinary chloride level, which fell steadily from 48 mEq./litre on the day of admission to 16 mEq./litre three days later (highest P:U ratio=5.5), suggested continued tubular ability to reabsorb chloride.

Chloride.—The general pattern was a rapid fall to very low values (1 to 20 mEq./litre) within 12 hours of burning after which the low chloride excretion continued for days. This occurred in patients with and without severe azotaemia.

In Case 12 (Fig. 7) the chloride fell to 15 mEq./litre by six hours after burning and then was between 4 and 15 mEq./litre for four days. The chloride concentration was independent of the sodium and potassium levels; it fell as the sodium and potassium concentrations rose and remained low when the sodium concentration subsequently fell. Chloride excretion was similar in Case 14 (Fig. 5) even though severe azotaemia and proximal tubular necrosis developed and the patients differed in therapy, age, and clinical course. In the severely oliguric Case 7 the chloride urinary level was maintained between 13 and 33 mEq./litre after the first 24 hours (Fig. 6).

Bicarbonate and Organic Acid Excretion.—Our colleague Miss S. Baar showed that when there was a high-sodium, high-potassium, and low-chloride excretion the apparent deficiency in anions (175 to 250 mEq./litre in Cases 12 and 14

was largely made up by bicarbonate, and substantial amounts of citric acid, ketoglutaric acid, and hippuric acid were also present.

Tubular Reabsorption of Water and Sodium.—The amount of water reabsorbed by the tubules in a given time may be expressed as a percentage of the glomerular filtration rate ($\frac{U}{P}$ creatinine). If the urine volume V is expressed as a percentage of this ($V \times \frac{P}{U} \text{creatinine} \times 100$), the formula $\frac{P}{U}$ creatinine $\times 100$ is obtained. This represents the fraction of filtered water excreted as urine. One hundred per cent. minus this value is the amount reabsorbed by the tubules, but for convenience the fraction excreted will be used. Normally this is about 1% in adults and 2% in children so that 98%–99% of the filtered water is reabsorbed by the tubules. A definite increase in $\frac{P}{U}$ creatinine above 2% indicates a diminished tubular reabsorption of water. This may result from an inhibition of antidiuretic hormone (ADH) activity or from osmotic tubular diuresis or from nervous causes.

The amount of sodium reabsorbed by the tubules may be expressed as a percentage of the amount filtered by the glomeruli in the same period (Platt, 1950). The latter is the plasma sodium level (PNa) × the G.F.R. or $\frac{U}{P}$ creatinine. If the amount of sodium in the urine (urine sodium concentration × volume or UNa × V) is expressed as a percentage of the amount of sodium filtered by the glomeruli ($PNa \times \frac{U}{P}$ creatinine) the percentage of filtered sodium excreted in the urine is $\frac{U}{P}$ Na $\times \frac{P}{U}$ creatinine $\times 100$. One hundred per cent. minus this value is the amount reabsorbed by the tubules, but for convenience the fraction excreted will be used. Normally this varies from about 0.5% to 1% so that more than 99% of filtered sodium is normally reabsorbed. Values lower than 0.5% indicate an increased tubular rate of reabsorption of sodium, and this is mediated by adrenal hyperactivity, particularly aldosterone secretion and nervous factors.

Platt showed that the percentage of filtered sodium excreted in the urine increased as the plasma creatinine concentration rose, that is, as the glomerular filtration decreased, presumably as a compensatory mechanism so that a "normal" percentage (0.5% to 1%) in these circumstances probably indicates an increased rate of tubular reabsorption of sodium.

The values for water and sodium excretion were available in 12 patients (Cases 4, 6, 7, 9 to 17) (Figs. 2 and 3). The patterns of excretion of filtered sodium and water behaved independently, although in four patients (Cases 7, 9, 12, and 14) there were early brief phases of parallel increased excretion. Simultaneous values of high percentage excretion of filtered water and low values for sodium occurred and indicated differential tubular activity of a high degree. Such divergent patterns were present in non-azotaemic subjects during the shock period and persisted in all the severely azotaemic patients whether or not tubular necrosis was present.

Water.—Two main patterns (A and B) in the tubular handling of water were found.

A. In five patients (Cases 4, 6, 9, 12, and 15), all without persistent azotaemia, there were episodic increases in the fraction of filtered water excreted during the first two or three days (Fig. 2). The peak values varied from 2% to 15% ; each episode lasted four to 12 hours and in four patients normal values were found between the peaks.

B. In the other seven patients, all dying with severe azotaemia, the fraction of filtered water excreted as urine was raised throughout most of the period of study (Figs. 3 and 4). In Cases 7, 10, 11, 13, and 17 there was a progressive increase in the fraction excreted during the first week after burning or longer, reaching maxima of 5% to 15% ; whilst in Cases 14 and 16 there was a peak excretion of filtered water during the first or second 24 hours (maximum figures 8.3% and 7.1% respectively) and a subsequent fall, but the terminal figures were still raised (5.5% and 3.1%).

In general there was a tendency for the percentage of filtered water excreted to increase as the G.F.R. decreased. In those with no or a temporary azotaemia the percentage was greatest during the period of the low G.F.R. The inverse relationship was best marked in the severely azotaemic subjects, not only in patients with a normal or relatively normal urine ouput (e.g., Fig. 3, Cases 10 and 11), but also in those in whom oliguria was severe (Fig. 3, Cases 7 and 17). The graphs showing percentage excretion of filtered water generally paralleled the rising curves of plasma urea and creatinine. In non-oliguric patients the relatively normal urine flow in the face of a reduced G.F.R. was the result of a decreased tubular absorption. In those who develop severe oliguria the increase in fractional excretion is insufficient to balance the decrease in filtration.

Sodium.—The excretion of filtered sodium during the first 24 hours was in two main patterns (A and B) ; subsequently a normal or lower than normal fraction was excreted in almost every patient.

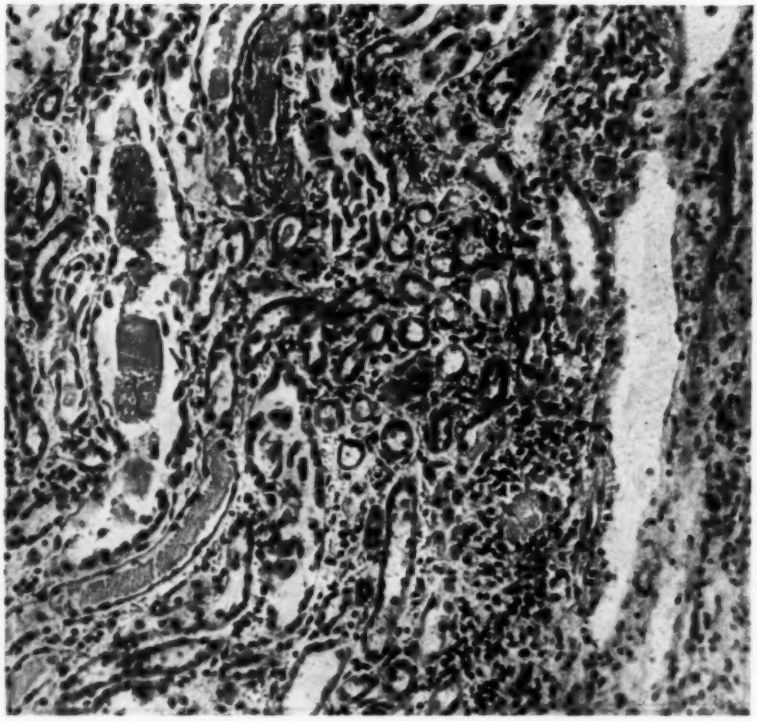

FIG. 8.—Boundary zone of the kidney in Case 10 showing diffuse distal tubular necrosis. On the right many Henle tubules are distended by haemoglobin casts and the tubular epithelium is thinned and necrotic in places. On the left there is a lymphocytic infiltration around the venule partly related to extruded casts. Haematoxylin and eosin, × 210.

A. In seven patients (Cases 6, 10, 11, 13, 15 to 17) the percentage excretion of filtered sodium was normal or decreased from soon after burning until death or the end of the study (Figs. 2, 3, and 4). The terminal values in the patients with severe azotaemia ranged from 0.06% to 1.3% (Case 10= 0.6% ; Case 11=1.3% ; Case 13=0.19% ; Case 15=0.06% ; Case 16=0.09 ; and Case 17= 1.8% at 24 hours after burning) which indicated increased reabsorption of sodium by the tubules.

B. In the other five subjects (Cases 4, 7, 9, 12, and 14) (Figs. 2 and 3) the percentage excretion of filtered sodium was increased for one or more periods during the first 24 hours with peak values rising to between 6% and 8% ; the fraction then decreased to low or normal values. The terminal values in the patients with severe persistent azotaemia were 2.3% (Case 7) and 0.4% (Case 14). Case 14 differed in that a fall preceded and succeeded a relatively prolonged increase in percentage excretion.

The relationship between the pattern of excretion of filtered sodium and the pattern of development of a low urinary sodium level was close but not absolute because the percentage excretion is also dependent on the G.F.R. Five out of seven patients with type A fractional excretion quickly developed a low urinary sodium level, whilst in four out of five of those with type B fractional excretion the low sodium concentration was preceded by a phase of sodium diuresis.

The most significant finding was the increased reabsorption of sodium not only in patients with and without severe azotaemia but also in those with tubular necrosis.

Morphological Changes

Glomerular and tubular changes are found in the kidneys of many patients who die after burning (Sevitt, 1956a and b ; 1957).

Tubular Necrosis.—This may be divided into (1) distal tubular necrosis (D.T.N.) (Fig. 8) and (2) proximal tubular necrosis (Figs. 9 and 10).

The former is characterized by the precipitation of casts, generally containing much haemoglobin, in the distal and collecting tubules and by tubulo-epithelial foci of necrosis or rupture apparently related to pressure or blockage by the casts. An

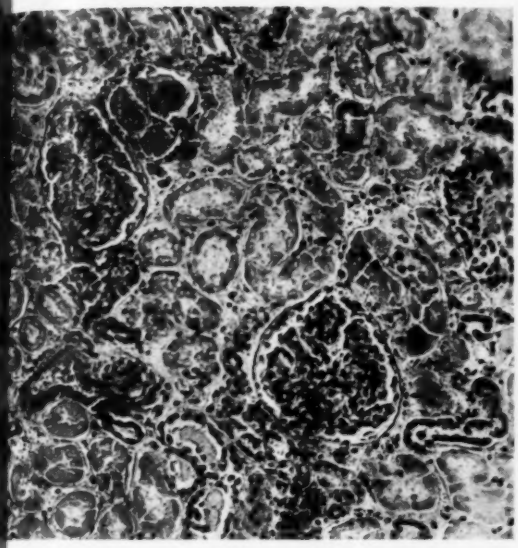

FIG. 9

FIG. 9.—Cortex of kidney in Case 11 showing extensive proximal tubular necrosis. Focal distal tubular necrosis is also present as shown by the distension and blockage of a distal convoluted tubule by a haemoglobin cast (top, left of centre). Haematoxylin and eosin, × 160.

FIG. 10.—Cortex of kidney in Case 14 showing extensive proximal tubular necrosis. Haematoxylin and eosin, × 205.

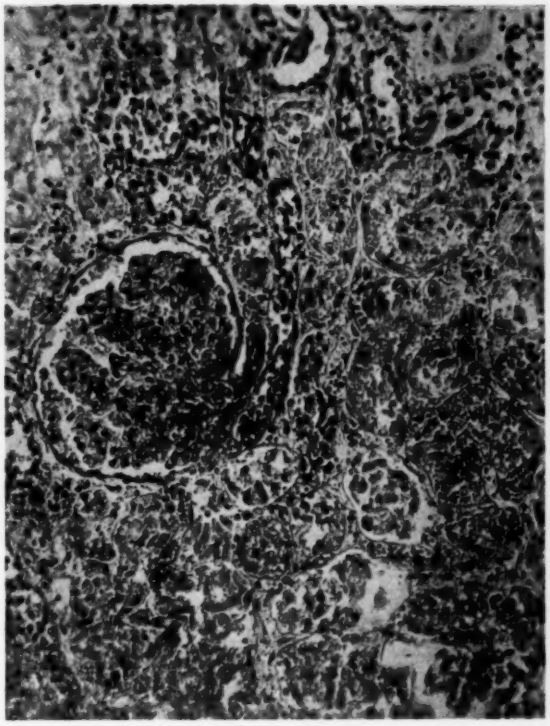

FIG. 10

inflammatory cellular reaction develops around extruded casts, interstitial oedema occurs, and mural venous thrombi and other evidence of tubulo-venous anastomoses are found. Two varieties are recognized, diffuse distal tubular necrosis when the condition is widespread, and focal distal tubular necrosis when few nephrons are affected.

Proximal tubular necrosis (P.T.N.) is characterized by extensive necrosis of the proximal convoluted tubules, but the distal and collecting tubules in the medulla appear histologically viable.

In the present series 10 of the 11 fatally burned subjects came to necropsy, and the individual results are outlined in Table II. There were three patients without tubular necrosis, two with focal distal tubular necrosis, three with diffuse distal tubular necrosis, and two with extensive proximal tubular necrosis, one of whom also had a focal distal tubular necrosis. In Table IV the patients are divided into four groups according to the development of azotaemia, the reversibility or persistence of the fall in the G.F.R., and the presence or absence of significant oliguria. In each group and in at least nine of the 10 patients with kidney histology available the urinary electrolyte pattern indicated continued tubular activity or hyperactivity and discriminating power. Nevertheless, severe azotaemia with or without oliguria was present in the three patients with diffuse distal tubular necrosis (Cases 8, 10, and 17). One of the patients with focal distal tubular necrosis had severe azotaemia and oliguria (Case 5) and the other had had a phase of moderate but temporary azotaemia without oliguria (Case 15). Both of the patients with extensive proximal tubular necrosis (Cases 11 and 14) developed a severe non-oliguric azotaemia. The other three subjects (Cases 1, 13, and 16) showed no evidence of tubular necrosis, but in two of them (Cases 13 and 16) the G.F.R. was low and severe azotaemia developed. They died nine and four days after burning when tubular damage would have been easily detectable had it been present. Neither patient had haemoglobinuria, and this may be significant, since

TABLE IV
RENAL FUNCTION AND TUBULAR NECROSIS

Azotaemia, G.F.R., and Urine Flow	Tubular Activity	No. of Deaths per Total No. of Patients	Renal Tubular Necrosis (No. of Patients)
No azotaemia; temporary fall in G.F.R.; no significant oliguria	Low urinary Na and Cl, high K	1/4	None
Moderate reversible azotaemia; temporary fall in G.F.R.; no significant oliguria	Low urinary Na and Cl, high K	1/4	Focal D.T.N.
Severe irreversible azotaemia; fall in G.F.R. early, severe and persistent or early moderate with subsequent decline; no significant oliguria	Low urinary Na and Cl, high K	6/6	Diffuse D.T.N. (2) Proximal tubular necrosis (2)† None (2)
Severe irreversible azotaemia; fall in G.F.R. early, severe and persistent; severe oliguria or anuria	Low urinary Na and Cl, high K (1 case); atypical excretion pattern of electrolytes (1 case)	*3/3	Focal D.T.N. (1) Diffuse D.T.N. (1)

* Necropsy refused in one patient. † One also had focal D.T.N.
Note: (1) The G.F.R. results refer only to 12 patients (see text).
(2) In some patients the low urinary sodium was preceded by a phase of sodium diuresis (see text).
D.T.N. = distal tubular necrosis.

a close relationship between this and distal tubular necrosis was previously reported (Sevitt, 1956a and b). It appears that the common association of tubular necrosis with severe azotaemia and a low G.F.R. is due to common pathogenetic factors; the inconstant relationship indicates that one does not cause the other.

Hydropic Tubular Change.—This affected the proximal convoluted tubules in three of the 10 patients. The condition, which was focal in Case 10, quite extensive in Case 17, and particularly well marked in Case 16 (Fig. 11), was not related to tubular necrosis. The affected epithelial cytoplasm was swollen and had a pale, empty appearance not due to lipoid or glycogen. Close examination showed a sparse fine eosinophilic structure in the cytoplasm as if various cellular structures were separated by an excess of fluid. The nuclei were generally basally situated and the cell borders thickened as if compressed by fluid.

Relationship to Lactosuria

Hydropic tubular swelling does not appear to be a degenerative phenomenon. It can be produced by the administration of sucrose, lactose, and other saccharides, and is probably an osmotic effect related to a disturbance in the normal osmotic relationship between the filtrate and tubular epithelium (Lamy, Mayer, and Rathery, 1906; Helmholz, 1933; Wilmer, 1944). Lactosuria, which may follow the application of penicillin-lactose powder to burns, was found in three of the eight patients studied (Cases 10, 11, and 16), in two of whom hydropic change was present. Lactosuria was not tested for in the other patient with hydropic tubules (Case 17).

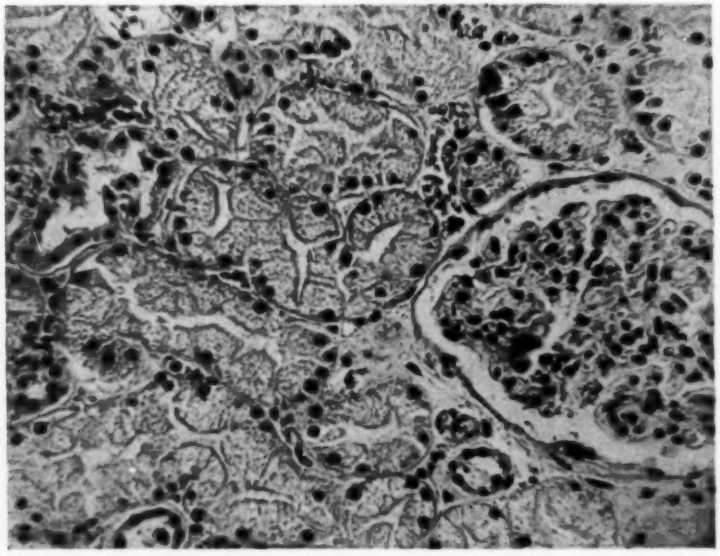

FIG. 11.—Cortex of kidney in Case 16 showing hydropic change in the proximal convoluted tubules. Haematoxylin and eosin, ×270.

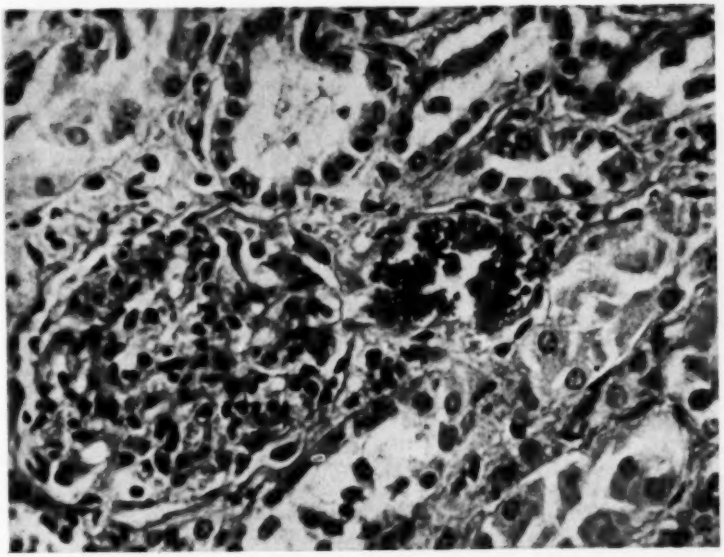

FIG. 12.—Cortex of kidney in Case 8 showing multiple hyaline (P.A.S.-positive) droplets or granules in the epithelial cytoplasm of the neck of a proximal convoluted tubule. Periodic-acid-Schiff and haematoxylin, × 360.

A possible relationship to a decreased tubular absorption of water was considered but was impossible to evaluate because the condition is known to be reversible. Its appearance was not necessarily related to the presence or absence of oliguria.

Hyaline Droplet Change.—This affected the neck-like origin of some or many proximal convoluted tubules of three subjects (Cases 8, 13, and 16) (Table II). The cytoplasm contained multiple fine or coarse eosinophilic hyaline droplets or granules (Fig. 12) ; these were periodic-acid-Schiff

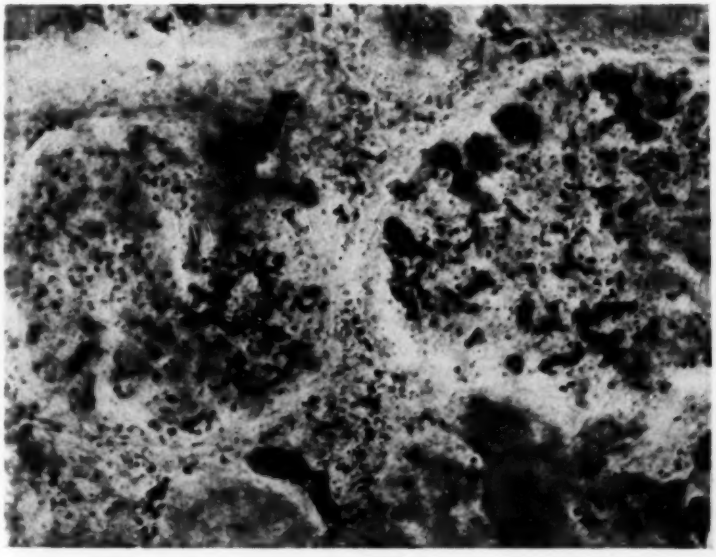

FIG. 13.—Two glomeruli in Case 17. These contain numerous fine droplets of fat (black dots) which are outside the capillary lumina and within epithelial and possibly endothelial cells. Frozen section of kidney stained by oil-red-O for lipid, × 350.

positive and probably protein in nature containing a muco- or glycoprotein. Their presence was not necessarily related to tubular necrosis. Similar droplets may be found in many conditions, including nephritis, nephrosis, eclampsia, and gastro-enteritis in children, but their origin is debatable. They might be tubulo-degenerative in origin (Allen, 1951), related to the reabsorption of filtered protein (Smetana, 1947; Oliver, Straus, Kretchmer, Dickerman, and Cherot, 1955), or possibly to reabsorption of glomerular glycoprotein released by adrenocortical hyperactivity (Masson, del Greco, Corcoran, and Page, 1953). A relationship to plasma transfusion cannot be close because it was absent in most of the present patients.

Lipid Glomerular Droplets. — Multiple fine droplets of fat are often seen in the glomerular tufts of burned patients when appropriately stained frozen sections are examined (Fig. 13). The droplets, which are generally 1–3 μ in diameter, appear as a fine dusting within the epithelial cells, some of which are closely packed with numerous globules. They are possibly also within endothelium but are rarely found within capillary lumina. Their density may vary from a heavy dusting (+ + +) to moderate numbers (+ +) or a few scattered droplets (+).

In the present series a + + + or + + degree of change was found in five subjects (Cases 11, 13, 14, 16, and 17) all of whom had severe azotaemia; few (+) or no droplets were found in the other five (Cases 1, 5, 8, 10, and 15), three of whom had severe azotaemia, one a reversible azotaemia, and one no azotaemia (Table II). The condition is not directly related to tubular necrosis because it was present in two patients without this condition (Cases 13 and 16) and was heavy, moderate, or slight in subjects with proximal or diffuse distal tubular necrosis.

DISCUSSION

Tubular Function and Tubular Necrosis

The results summarized and correlated in Tables II and IV show that persistent tubular activity was found whether or not the patient developed azotaemia, whether or not the azotaemia and the fall in the G.F.R. were temporary or persistent, whether or not oliguria occurred, and irrespective of the character and extent of tubular necrosis. The latter was often found in azotaemic patients but not invariably: two patients without tubular necrosis became severely azotaemic and another with only a focal distal tubular necrosis developed severe oliguria. The converse, diffuse distal tubular necrosis without azotaemia, has been previously reported (Sevitt, 1956a). The previous finding of diffuse distal tubular necrosis in severely oliguric and non-oliguric subjects has been confirmed; and it is now found that extensive proximal tubular necrosis may occur without oliguria. The results mean that tubular necrosis cannot be the pathological basis of acute renal dysfunction after burning. Both are often present because they have common pathogenetic factors. This view is contrary to the general belief that the tubular lesion or lesions termed by different workers "lower nephron nephrosis," "pigment nephrosis," "acute tubular necrosis," "tubulorhexis," and "nephrotoxic lesions" impose a characteristic pattern of renal failure (Lucké, 1946; Bull, Joekes, and Lowe, 1950; Bull and Dible, 1953; Oliver, MacDowell, and Tracy, 1951; Oliver, 1953). Bull *et al.* (1950) described a period of anuria or severe oliguria followed by early and late diuresis. The urine in the oliguric and early diuretic phases was said to approximate to a plasma filtrate; the P : U ratios for sodium and chloride were low and the clearance values for creatinine, para-aminohippuric acid, and other substances were greatly reduced. They attributed these findings to unselective back diffusion and explained dysfunction in terms of morphology, correlating oliguria with tubular necrosis and early diuresis with regenerating tubular cells. Correlation, however, does not prove a causal relationship. Their cases and those of other workers were selected because of severe oliguria, but in our experience with burned patients oliguria is less common than the non-oliguric form of acute azotaemia and is merely part of the spectrum of renal dysfunction. The interpretation of some of the reported findings is not above criticism. For example, the hyperexcretion of potassium in the so-called early diuretic phase is surely a manifestation of intense tubular activity.

Our results indicate that many nephrons continue to function even when tubular necrosis is histologically extensive. The damaged nephrons are excluded from the urine flow, but the remainder continue to function and determine the composition of the urine. This concept centres the renal problem not on tubule dysfunction but upon glomerular filtration and on the size and reserves of the functioning tubular mass.

The pattern of electrolyte excretion is independent of tubular necrosis. It is related to the hormonal response of the body to trauma, the deviation of sodium and chloride into the burn oedema, and their modifications by fluid therapy. There is ample evidence of prolonged adreno-

cortical hyperactivity in severely burned patients (Harkins and Long, 1945; Sevitt, 1951, 1954, 1955; Evans and Butterfield, 1951; Symington, Currie, Curran, and Davidson, 1955), mainly related to glucocorticoid secretion (Tompsett and Oastler, 1947; Hume, Nelson, and Miller, 1956). Although aldosterone output in burns has not been reported the low urinary Na/K ratios (Llaurado, 1955) in our patients suggest that there is an increased secretion of this hormone. The water and electrolyte disturbance and hormonal responses in burned patients differ qualitatively and quantitatively from the changes in unburned patients; this may partly explain the differences in our results from those of other workers, most of whose patients developed severe oliguria after traumatic shock, abortion, haemolytic transfusion reactions, and other conditions unrelated to burning.

The aetiology of tubular necrosis after burning has been discussed elsewhere (Sevitt, 1956b, 1957). Both the fall in G.F.R. and tubular necrosis have common pathogenetic factors. Renal vasoconstrictive ischaemia and the precipitation of haemoglobin casts from oligaemia and haemoglobinaemia may be followed by distal tubular necrosis, whilst renal vasoconstrictive ischaemia and a redistribution of the intrarenal blood flow (aided by advancing age and pre-existing nephrosclerosis) may be followed by proximal tubular necrosis.

Oliguria and Hyperpotassaemia.—Hyperpotassaemia regularly develops in patients with acute oliguria and is said to be related to the renal inability to excrete potassium because of tubular necrosis. Our findings indicate that oliguria is the important factor and not tubular necrosis; a significant hyperpotassaemia was found only in the three severely oliguric patients (Cases 5, 7, and 17) who showed either a rapid or progressive rise to 6.1, 7.3, and 6.9 mEq. of potassium per litre of plasma respectively. The non-oliguric subjects, including those with proximal or diffuse distal tubular necrosis, had normal plasma potassium levels (3.6–5.0 mEq./litre); three of them had phases of hypopotassaemia lasting one to three days.

Oliguric and Non-oliguric Azotaemia.—Correlation of the G.F.R. with the rate of excretion of urine has shown that the proportion of filtered water passed as urine is often much increased; overall it is inversely related to the G.F.R., particularly in those with a persistent fall in filtration and a severe azotaemia. The reduced tubular reabsorption of water was found in those with severe oliguria as well as in those who passed normal or polyuric volumes. It is evident that the relatively normal or increased urine volume in the face of a reduced G.F.R. is dependent on a sufficiently reduced tubular reabsorption of water; oliguria occurs when the decrease in tubular reabsorption is insufficient to balance the decrease in filtration.

The reduced reabsorption might be interpreted as an impaired tubular function, but it would then be a selective impairment because other absorptive and excretory powers seem hyperactive. It may be compensatory to the low G.F.R., since the direct relationship between the percentage of filtered water excreted and the plasma creatinine (or urea) is analogous to the compensatory sodium-excretion mechanism described by Platt (1950) in chronic renal failure.

The decreased reabsorption of water might be mediated by a decreased secretion of pituitary anti-diuretic hormone and/or an increased secretion of adrenal 17-hydroxycorticosteroids. Baar (1956) found that the peptide fraction isolated from the pooled urines of burned patients with reduced urine flows had little anti-diuretic activity. However, it is not known whether the decreased excretion of anti-diuretic hormone occurs only in those with a reduced tubular reabsorption of water. Anti-diuretic hormone secretion is known to be inhibited after expansion of the extracellular space by intravenous normal saline (Strauss, Davis, Rosenbaum, and Rossmeisl, 1951) and might be mediated by the oedematous burn which is greatly expanded by fluid therapy. The increased plasma level of 17-hydroxycorticosteroids found by Hume *et al.* (1956) could contribute to the decrease in tubular reabsorption of water since an antagonism between the action of anti-diuretic hormone and hydrocortisone is known (Graber and Beaconsfield, 1955; Raisz, McNeely, Saxon, and Rosenbaum, 1958). A possible contributory cause is osmotic diuresis from lactosuria aided by the temporary glycosuria which is a feature of extensively burned patients.

Fall in Glomerular Filtration

The G.F.R. invariably fell considerably during the first 12 hours after burning even though transfusion therapy was most intense during this time. In some patients, mainly children, the fall was temporary and normal filtration was re-established by 24 to 72 hours; in others, mainly adults, the low rate of filtration persisted or even further decreased until death supervened. The influence of age on renal function is similar to that found previously when azotaemia was used as the index of glomerular function (Sevitt, 1956a). The

different response of children to that of adults may represent a renal haemodynamic resiliency which is deficient in the higher age group. These patterns of change in the G.F.R. are similar to those described by Dziemian (1948) in both treated and untreated burned goats, but they are in conflict with the results of Haynes, DeBakey, and Denman (1951), who reported normal or higher than normal rates of filtration in severely burned patients. Their studies are confined to patients with high urine flows (120 ml. per hour or more) and their clearance results were based on only two 20-minute urine collection periods. The rapid fluctuations found by us indicate the danger of drawing conclusions from one or two estimations, and also indicate that the normal mechanism stabilizing glomerular filtration has been impaired even in those without azotaemia.

Pathogenesis.—The main problems are (1) the cause and mechanism of the initial fall of the G.F.R. after burning, and (2) the changes which can prevent a return to normal filtration in patients receiving early and energetic transfusion.

(1) The Initial Fall in G.F.R.—The available evidence indicates that the major mechanism is a severe renal vasoconstriction which reduces the glomerular blood flow and so influences filtration. Normally the kidneys received 20–25% of the cardiac output, but Lauson, Bradley, and Cournand (1944) reported that in patients with traumatic shock (also one burned patient in shock) the fraction is greatly reduced. A severe reduction in cardiac output is accompanied by a proportionately greater fall in the renal blood flow often to 10% of the normal. This must be due to a selective renal vasoconstriction. Studies in animals and in man have shown that extensive burns are also followed by a major fall in cardiac output at least partly related to the development of oligaemia (Richards, 1943–44; Hardy, Neely, Wilson, Lovelace, and Jabbour, 1955; Dobson and Warner, 1955; Gilmore and Handford, 1956). At first the blood pressure remains normal or even rises, indicating a marked increase in peripheral resistance.

Renal vasoconstriction may also be produced by humoral agents, among which are noradrenaline, which is known to be excreted after burning (Goodall, Stone, and Haynes, 1957), the vasoconstrictive substance in plasma described by Page (1943), and substances released from lysed red cells and platelets, possibly including 5-hydroxytryptamine.

Glomerular filtration may also be retarded by a phase of therapy-induced hyperproteinaemia (8 to 9.5 g. per 100 ml.) in some patients transfused with plasma containing about 5 g. protein per 100 ml. (Graber, unpublished, quoted by Sevitt, 1957).

(2) Irreversible Fall in G.F.R.—A return to normal or near-normal kidney function after early and energetic fluid therapy occurred in only eight out of the 17 patients, whilst in the others the low G.F.R. was unaffected. This ability to respond to therapy seems related to the age of the patient and the extent of burning. None of the four patients, adults or children, with burns involving less than 30% of the body surface developed an irreversible renal failure, but the latter occurred in all the seven more extensively burned adults and in two of the six more extensively burned children.

We do not know the cause of the persistent fall in filtration, but the following facts and speculations are worthy of consideration.

Although Hardy and his colleagues (1955) found that intravenous therapy in burned patients was followed by a prompt improvement or restoration of the cardiac output, this was not the experience of Gilmore (1957) in burned dogs. He found that restoration of plasma volume did not always produce a normal cardiac output, nor did a normal cardiac output necessarily indicate restoration of plasma volume. In addition restoration of cardiac output may not be followed by a normal distribution of blood flow which could be deviated from the kidneys into the acutely inflamed burned area and into the congested gastro-intestinal tract and spleen.

It is also possible that the reduced renal flow might promote the renal secretion of "renin" or an allied substance which could then perpetuate the vasoconstriction.

Tubular necrosis must reduce the functioning nephron mass and contribute to the reduction in the G.F.R. Oliver *et al.* (1951) pointed out that necrosis, rupture, or blockage by a cast, even when localized to part of a tubule, would interfere with the functioning of the whole nephron. The development of interstitial oedema in kidneys with tubular necrosis has suggested that there is a rise in intrarenal pressure which might reduce intrarenal blood flow after filtration and produce oliguria. However, de Wardener (1955) failed to demonstrate a rise in intrarenal tension in the kidneys of dogs with necrosis produced by ligation of the renal artery, and Munck (1958) also failed to demonstrate a rise in tension in five patients with oliguric failure after wedging the tip of a catheter within a renal vein.

Glomerular Changes. — Tubular necrosis cannot be the whole explanation, because it was not found in two patients with an irreversible fall in filtration. Moreover, Munck (1958) recently found that the degree of fall in the creatinine clearance is far greater than can be accounted for by the reduction in renal blood flow. In six patients with oliguric failure the average renal blood flow (measured after inhalation of radioactive keypton and renal vein catheterization) was reduced to about one-third of normal whilst creatinine (G.F.R.) was reduced to 1% or less. This suggests to us that changes within the glomeruli may play a part in the production and maintenance of a low filtration rate.

It is the common belief that there is no histological abnormality of the glomerulus which can explain acute renal failure, but our finding of large numbers of fine fatty droplets in the glomerular epithelium and possibly in the endothelium of the tuft makes reconsideration of this necessary. Electronmicroscopic studies of the normal glomerulus (Pease, 1955) show that the capillary endothelium is full of fine pores and that the epithelium has multiple fenestrations between fine foot-like projections cemented to the basement membrane. This indicates that the only glomerular structure capable of acting as a filter is the basement membrane. It is worth speculating whether subtle glomerular changes, difficult to define in ordinary histological sections, such as acute swelling of the epithelial or endothelial cytoplasm, may be present in patients with a persistent fall in the G.F.R. Such changes would reduce the porosity of the cells apposed to the filtering membrane. In this connexion the appearance of fatty droplets in the epithelium may be a histological sign of a more serious and subtle change affecting filtration. Changes in the glomerular basement membrane itself are also worthy of study.

SUMMARY AND CONCLUSIONS

Renal function was studied in 17 extensively burned patients admitted and treated by intravenous therapy by one to three and a half hours after burning and was related to the renal histological changes in those who subsequently died. Patterns of glomerular and tubular function were established from the examination of serial specimens of urine, hourly at first, and of appropriate blood samples; from these estimates were made of the glomerular filtration rate (creatinine clearance), urea clearance, plasma creatinine and urea levels, and of the excretion of water, sodium, potassium, and chloride.

Evidence is presented that non-selective back diffusion plays no part in the composition of the urine in patients with acute renal failure and that therefore the G.F.R. could be measured by creatinine clearance.

The G.F.R. fell considerably during the first few hours after burning even though transfusion with plasma was most intense at this time. In some patients, mainly children, recovery occurred by 24 to 72 hours and azotaemia was absent or moderate and temporary. In others, mainly adults, the low G.F.R. persisted or even worsened until death supervened; in them azotaemia was considerable, severe oliguria sometimes developed, but more often the urine flow was normal, near-normal, or even polyuric.

A diminished tubular reabsorption of water was found in oliguric and non-oliguric patients with severe azotaemia; when this balanced the low rate of filtration the urine flow was normal whilst oliguria occurred when the fall in the G.F.R. was greater.

Similar patterns of tubular activity (including the maximal power to reabsorb sodium and chloride and to excrete high levels of potassium and a tubular ability to differentially handle urea, creatinine, water and electrolytes) were found in patients whether or not they developed azotaemia, a persistently low G.F.R., or oliguria.

Similar tubular activity was found in those who survived and in those who died with proximal or distal tubular necrosis or without tubular necrosis.

The results conflict with the concept that acute renal failure is characterized by loss of tubular function, that it is due to renal tubular necrosis, and that severe oliguria or anuria is always present. The major dysfuncion is glomerular and not tubular, even though tubular necrosis and blockage by casts are often found. The aetiological factors relating to glomerular and tubular function are discussed. Attention is drawn to a possible structural change in the glomeruli as the cause of the persistent fall in the G.F.R. and is focused on the functioning nephron mass and reserves to explain the composition of the urine.

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SOME EFFECTS ON BLOOD COAGULATION OF ERYTHROCYTES AND OTHER CELLS

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The activity of intact cells or of extracts from cells may be studied in blood coagulation systems to elucidate the problems of coagulation or to investigate the properties of cells and their extracts. The present report is concerned primarily with the latter objective.

Since the beginning of this century aqueous extracts of cells have been known to accelerate clotting. In 1916 McLean prepared a protein-free accelerator from brain by extraction with lipid solvents. Crosbie and Scarborough (1941) reported that the clotting time of plasma when Russell viper venom was used was accelerated by the haemolysis of red cells. They suggested that this acceleration might be due to a highly active phospholipid. Quick, Georgatsos, and Hussey (1954) also reported the coagulant activity of red cell haemolysates, and Hussey and Kaser (1956) prepared an active phospholipid extract of red cell stroma and called it erythrocytin.

If platelets are removed by centrifugation from citrated plasma, on recalcification this plasma clots badly ; if platelets are added to such plasma, the clotting process reverts to normal. Using platelet-poor plasma as a substrate, substances of known chemical structure or tissue extracts or intact cells may be added in order to see what will correct the defect as completely as platelets themselves. Material which can substitute for platelets will be called " active " and that which has no effect will be called " inactive." A chemical substance or tissue extract which exhibits full activity is almost certain to contain cephalin-like molecules, and these must be either free or " available " at the surface of a larger molecule (O'Brien, 1958). It is still possible that there are chemical configurations other than the cephalins that could mimic the action of platelets, but the definition of " activity " remains and is a useful concept.

This report is not immediately concerned with the thromboplastic substance found, for example, in a saline extract of brain, which has properties quite different from those of platelet-lipid, but such a brain extract contains active lipids (McLean, 1916) and these are " available " for tests able to detect platelet-like activity. In brain extracts the active lipid is combined with a protein and both components seem to play a part in determining its thromboplastic power (Mills, 1921 ; Chargaff, Ziff, and Cohen, 1940). In studying some tissue extracts advantage was taken of the species specificity (Trevan and Macfarlane, 1936) of the brain-like activity to exclude it. Many human tissue extracts possess this activity in varying degree when tested in human plasma. Guinea-pig tissue extracts, when tested in human plasma, exhibit platelet-like activity but no brain-like activity.

Experimental Observations

Methods.—The methods of Biggs and Macfarlane (1957) were used throughout, and the tests employed, which are all very sensitive to platelet deficiency, were as follows: the plasma clotting time, the prothrombin consumption index, the thrombin generation test, the thromboplastin generation test, and the " stypven " time, which is especially sensitive to the addition of small quantities of active lipid (O'Brien, 1956). A suspension of " asolectin," a phospholipid extract of soya beans, 1 mg./ml. saline, was frequently used as an easily controlled substitute for platelets.

Red Cells.—One volume of fresh, intact, washed, human red cells in saline was added to platelet-poor plasma and the mixture was tested in all the above-mentioned tests; it was found that this addition did not improve the performance when compared with the saline control. Red cells may, therefore, be called inactive. A sample of these washed red cells was haemolysed by the addition of distilled water and the red cell ghosts were washed 10 times in water by repeated centrifugation at 4,000 r.p.m. for 15 minutes. The resulting particulate grey deposit, free of haemoglobin, was regularly found to be active in these tests. Particulate material after haemolysis by freezing and by ultrasonic agitation, after haemolysis by digitonin,

Clostridium welchii, α toxin, and other haemolytic agents, was more difficult to wash by centrifugation, but these unwashed, haemoglobin-containing haemolysates were also fully active in all tests.

Ghost red cells were prepared by the addition of 1 ml. of water to 1 ml. of packed washed red cells. The ghost red cells were pleomorphic but were usually pale biconcave discs, and they were always active. If the ghost red cells were centrifuged and resuspended in saline they again became inactive, and the sequence of activation and inactivation by suspending the haemolysed ghost red cells successively in water or saline could be repeated. The total ionic strength in any clotting mixture was inevitably lowered when red cell ghosts in water were added. The activity of isolated phospholipids was barely affected by such a change (Table I).

TABLE I
STYPVEN CLOTTING TIME OF RED CELLS VARIOUSLY TREATED

Test Substance	Clotting Time (sec.)
Red cells in saline + plasma + stypven + calcium	24
Red cell ghosts in water " " "	12
Red cell ghosts in saline " " "	20
Asolectin in water " " "	15
Asolectin in saline " " "	17

One volume of each material was used. Red cell ghosts in saline were prepared from the red cell ghosts in water by centrifugation, removal of the water, and the addition of an equal volume of saline.
Asolectin = a commercial mixture of active phospholipids, 1 mg./ml.
Stypven = Russell viper venom, 1 : 10,000 in water.
Calcium = calcium chloride, 0·025 M.

Other Cells.—It has already been reported that the stypven time of citrated whole blood is similar to that of platelet-poor plasma (O'Brien, 1956). Thus, intact platelets and white cells are inactive. The inactivity of intact platelets could not be investigated further, because in all other clotting tests the platelets break up in the early stages of coagulation. Live, washed HeLa* cells (a tissue culture explant from a human carcinoma of the cervix) and live, washed human sperms were shown to be inactive. No other live cells have been studied free from contamination with disrupted cells.

White cells (largely freed of platelets), platelets (largely freed of white cells), HeLa cells, and sperms were all killed and/or fragmented by freezing or boiling or ultrasonic agitation. The resulting suspensions always showed activity in whichever tests were used. The HeLa cells sometimes showed slight brain thromboplastic activity. About 1 g. of fresh guinea-pig lung, liver, muscle, brain, and fat was separately finely sliced and washed till grossly free from red cells. Each tissue was then ground up in 3 ml. of saline in a mortar. The saline extracts of all these organs were highly active.

*Dr. L. A. Hatch brought the HeLa cells of the BW line from the Virus Reference Laboratory, Colindale, to Portsmouth. Grown in lactalbumen Hanks solution with added human, horse, and calf serum, they were suspended by mechanical scraping.

Table II summarizes two typical sets of results. It will be seen that intact red cells were inactive throughout, and that live HeLa cells and sperms were almost completely inactive. When the extracts of guinea-pig tissues were added to guinea-pig plasma all the extracts (including human brain) had some thromboplastic activity, since all the clotting times were shorter than that caused by the addition of asolectin. But when added to human plasma they all accelerated the clotting time to about that caused by the addition of asolectin, a finding consonant with platelet-like activity. Under these conditions only human brain extract showed the typical thromboplastic activity. When the guinea-pig extracts were added to a mixture of human plasma and "stypven" the marked platelet-like activity was evident. All cell extracts showed marked activity in the thrombin generation test. In the thromboplastin generation test, damaged HeLa cells and sperms were active.

TABLE II
THE PLATELET-LIKE ACTIVITY OF CELLS AND CELL EXTRACTS IN CLOTTING TESTS

Test Substance	Guinea-pig Plasma + Calcium	Human Plasma + Calcium	Human Plasma + Stypven + Calcium	Thrombin Generation in Human Plasma*	Thromboplastin Generation†
Saline	210	230	23	87	
Asolectin	60	90	6	12	
Human intact R.B.C.s		235	22	88	
Human R.B.C.s fragmented ..		120	9·5	11·5	
Guinea-pig fat extract ..	41	128	7·5	13	
Guinea-pig liver extract ..	42	134	7	17	
Guinea-pig lung extract ..	26	110	6		
Guinea-pig muscle extract ..	30	120	7·5		
Guinea-pig brain extract ..	22	140	6	15	
Human brain extract	30	12	6	11	
Saline		125	23	125	27
Asolectin		45	7·5	12	7
Human HeLa cells (90% live) ..		60	19	120	
Human HeLa cells frozen		45	12	9	11
Human sperms (80% motile) ..		165	20	120	
Human sperms boiled		100	14	31	15

One volume of the test substance in saline was added to 1 vol. of plasma with or without 1 vol. of "stypven" as indicated, and the clotting times in seconds are recorded in the first three columns.
* The time in seconds reported is that of the shortest fibrinogen clotting time of any of the sub-sample tubes.
† This test is run normally, but the test substance was substituted for platelets. The shortest clotting time in seconds of any of the plasma sub-tubes is recorded.

Conclusions

It seems probable that all human cells when alive and intact are inactive in all clotting systems, an observation known for many years (see, for

example, Lister, 1863). The guinea-pig extracts were probably slightly contaminated with red cells and platelets, but the HeLa cells and sperms could not have been contaminated; it may be concluded that on death or disruption of all the cell types so far tested a considerable degree of platelet-like activity becomes manifest. Thus many cells are similar to platelets in exhibiting platelet-like activity on disruption. The unique property of platelets seems to be their ability under appropriate circumstances to release this activity into the blood stream.

The platelet-like activity of dead tissue cells may be related to the intercellular coagulation seen in infected tissues. In these circumstances plasma diffuses into the intercellular spaces. The damaged tissue cells, even if they do not possess brain-like thromboplastic properties, are now shown to possess platelet-like activity. Thus they may facilitate extravascular coagulation by virtue of this property. The platelet-like activity of the haemolysed red cells may be responsible for the thromboses in sickle cell anaemia and for the afibrinogenaemia following massive haemolysis due to an incompatible blood transfusion.

The red cell membrane is known to contain phospholipids, including cephalins, which on extraction exhibit platelet-like coagulant activity. Such activity is absent in intact red cells, is present in haemolysed red cell ghosts in water, and is again absent in red cell ghosts resuspended in saline. This suggests that in the first and last situations the cephalin molecules are so orientated that they are "unavailable" to clotting tests, but that in red cell ghosts suspended in water the cephalin molecules are differently, and reversibly, orientated. Evidence has already been presented (O'Brien, 1958) which suggests that cephalin molecules may be orientated in two ways in a protein molecule. Thus in some lipoprotein molecules the cephalin molecules are "available" to accelerate coagulation tests, and in other lipoproteins they are "unavailable."

The present findings combined with those of other workers suggest that three properties of cell membranes may be interrelated. These properties are osmotic activity, stickiness, and clotting activity. Teorell (1952) has shown that in red cell ghosts resuspended in saline the osmotic gradient is re-established. Bangham and Hancock (1955) have shown that dead sperms stick to glass beads but live sperms do not. It will be seen from Table III that, in all the situations studied, osmotic activity is associated with an absence of stickiness and with an absence of coagulant activity. The reverse is also true: a damaged osmotically inactive membrane is sticky and exhibits coagulant activity. If these three properties of membranes are truely interrelated it is possible that the osmotic properties and the stickiness of the cell as well as the coagulant activity of the cell are all related to the orientation of the cephalin molecules at the surface.

TABLE III

POSSIBLE RELATIONSHIP OF SOME PROPERTIES OF CELL MEMBRANES UNDER DIFFERENT CONDITIONS

	Good Osmometer (Physiologically Intact)	"Sticky" Cell Surface	Activity in Clotting Systems ("Available Cephalins")
Intact red cell	Present	Absent	Absent
Red cell haemolysed in water (ghost)	Absent	?	Present
Ghost resuspended in saline	Present	?	Absent
Live sperms	,,	Absent	,,
Dead ,,	? Absent	Present	Present
Intact native platelets	? Present	Absent	Absent
"Activated" platelets	?	Present	Present

Summary

Intact human red cells are inactive in blood coagulation tests. Red cell ghosts in water and washed particulate matter from the fragmented red cell membranes exhibit activity similar to platelet lipoid, and may therefore have "active" cephalin molecules suitably orientated at the surface. Red cell ghosts suspended in saline again become inactive. All other live intact cells are probably inactive. Fragmented, all cells examined have shown platelet-like activity. Some implications of these observations relating to the properties of the cell membrane are discussed.

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A TECHNIQUE FOR STUDYING THE ACTION OF ANTISEPTICS ON BACTERIA IN SUBCUTANEOUS TISSUES, WITH SPECIAL REFERENCE TO CHLORHEXIDINE

BY

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Bacteria which gain access to a freshly inflicted wound are at first external to the tissues and do not immediately cause any reaction. During this stage, which is commonly said to last up to about six hours, the wound is described as contaminated, and the organisms in it may be susceptible to antiseptics. Later, when bacteria have invaded the tissues, the wound is described as infected, and locally applied antiseptics are at a great disadvantage. The great differences which exist (and the difficulties in defining them) between accidental wounds in man and animals in respect of the age and nutritional status of the subject on the one hand, and the nature, site, severity, and degree of contamination of the wound on the other, have made the task of carrying out adequately controlled clinical trials of the value of antiseptics extremely formidable. In fact, only two such trials have been carried out, one by Williams and Miles (1949) dealing with several antiseptics, and a co-operative one carried out by groups of American workers on the value of locally applied sulphonamides reported by Meleney (1945). A study on a smaller scale, but carried out in great bacteriological detail, was reported by Florey, Ross, and Turton (1947).

These difficulties have led to several attempts to establish experimental wounds in which the degree of trauma and of bacterial contamination and the manner of treatment were controlled. In some methods bacteria have been injected subcutaneously or rubbed into superficial cuts, treatment being given on one or several occasions by swabbing or irrigation or by subcutaneous injection into or around the contaminated site (Browning and Gulbransen, 1925, 1928; Collier and Bernhagen, 1928; Hata, 1932; Morgenroth and Abraham, 1920; Reinhardt, 1922; Schiemann, 1922). Gordon, McLeod, Meyr-Harting, Orr, and Zinnemann (1947) removed an ellipse of skin, contaminated the raw area with streptococci, and treated it by dropping on antiseptic solutions. These methods have failed to control one or more of the following variables: (1) The number of organisms implanted in or on the tissues; (2) the amount of antiseptic in contact with the contaminated tissues; (3) the length of time for which the antiseptic acts. The third point is particularly difficult to meet, because any form of bandaging of small animals is almost impracticable. It is believed that the method described below, whilst not imitating the treatment of traumatic wounds, goes far towards controlling the above variables.

The present method is based upon two simple steps: (1) The subcutaneous injection of a small measured volume of a suspension of an organism capable of causing the death of the animal; (2) the injection of a somewhat larger volume of antiseptic into the same site shortly afterwards.

Since the site of injection of the organisms is practically bloodless, a closer approach to the conditions in a traumatic wound may be made by suspending the bacteria and dissolving or suspending the antiseptic separately in sterile, defibrinated blood. The degree of success of any antiseptic treatment is assessed by comparing the number of survivors in the treated and control groups (since a test organism is used which causes the death of all untreated animals at the dose injected) and the mean survival times of those which die in the two groups.

The method seems to be applicable to any experimental animal, but it has in fact been applied only to mice. The experiments have been referred to as relating to the treatment of "wounds" (in inverted commas because the method involves so little trauma).

Materials

Anaesthetic.—One part of a stock solution of tribromethanol in amylene hydrate containing 1 g. in 1 ml. ("avertin," Bayer Products Ltd., Eden Street, Kingston-on-Thames) was diluted in 39 parts of 10% ethyl alcohol. The dilute (2.5%) solution was made freshly for each experiment. This anaesthetic was found far superior to pentobarbitone for this purpose, because deaths from the anaesthetic did not occur.

Bacterial Suspension.—A stock culture of *Streptococcus pyogenes*, Kruger strain, in cooked-meat broth was kept in the refrigerator. For use, 0.5 ml. of the supernatant of this broth was added to 3 ml. of Wright's broth containing 5% of horse blood. This was incubated overnight at 37° C. and one part was diluted in 1,000 parts of plain broth or of 90% horse blood, according to need.

Antiseptics.—These were used as solutions or suspensions in water or in 90% horse blood (prepared by adding 1 volume of strong antiseptic to 9 volumes of blood). Where necessary, a non-toxic, non-ionic dispersing agent was used. The strength of the antiseptic solutions was limited to that shown in preliminary experiments to cause no obvious distress to the animals when 0.2 ml. was injected subcutaneously.

Methods

Basic Method.—Ten mice (18–22 g.) were anaesthetized by the intraperitoneal injection of 0.2 ml. of 2.5% tribromethanol (i.e., a dose of 5 mg./20 g.). Six minutes later all the mice were usually "sleeping" quietly. A No. 1 hypodermic needle was then inserted subcutaneously along the back for a distance of about 1 in. and 0.05 ml. of bacterial suspension was injected from a tuberculin syringe. The syringe was detached, leaving the needle *in situ*, and the mouse placed carefully on its side. A fresh needle was attached to the syringe, the plunger adjusted to the next mark and 0.05 ml. injected into the second mouse as before. The remaining mice were treated in the same way.

Twenty minutes after the first mouse had been infected it was treated with 0.2 ml. of antiseptic injected through the indwelling needle, taking care to alter its position as little as possible. All the mice were treated similarly in turn. After each mouse was treated, the needle was removed and the mouse returned to its cage. A group of control mice was injected with the bacterial suspension followed by saline.

Mice usually began to recover from the anaesthetic after about 30 minutes, and in two or three hours they were usually quite active. It was occasionally found that a mouse was not sufficiently under the influence of the anaesthetic at some stage. If so, the intraperitoneal injection of about 0.05 ml. of the anaesthetic solution almost always produced satisfactory relaxation in a minute or two.

Modifications of the Basic Method.—Three modifications for different effects were employed.

Addition of Blood.—To make the conditions a more severe test of the effectiveness of any treatment, both the infecting organism and the antiseptic treatment were prepared and injected in horse blood. This was done by preparing both the culture and antiseptic at 10 times the required strength and then adding 1 volume of each (separately) to 9 volumes of sterile horse blood. The other manipulations proceeded as described above. Control experiments showed that mice died at the same rate when the organisms were suspended in blood as when they were suspended in broth. The two types of experiment are distinguished in tables of results by showing the "vehicle" as "water" or "blood."

Comparison of Local with Systemic Action.—It was necessary in certain instances to decide whether the action of any particular antiseptic was exerted directly upon the organisms at the site of their injection, or was the secondary result of its absorption and systemic distribution. This was done by comparing two groups of animals, one treated as already described and the other "treated" locally with saline injected down the original needle, and at some remote site with the same dose of the same preparation as was used to treat the first group.

Delay in Treatment.—To demonstrate the effect of a delay in treating the subcutaneously deposited organisms, several groups of mice were anaesthetized and the organisms injected in the usual way. Successive groups were treated with antiseptic after the desired intervals, anaesthesia being maintained by occasional injections of small doses of tribromethanol, as required. Mice have been maintained under continuous anaesthesia in this way for six hours.

Results

An example of the results to be expected from the application of the technique described above is given in Table I, which shows the effects of

TABLE I

TREATMENT OF STREPTOCOCCAL "WOUNDS" WITH THERAPEUTIC AGENTS APPLIED LOCALLY AND SYSTEMICALLY

Treatment	Site of Treatment	Total Deaths	Mean Survival Time of Mice Dying (Days)
Penicillin G (0·07%, i.e., 1,150 units/ml.)	Local	9	3·1
	Remote	10	2·8
Streptomycin (0·5%, i.e., 5,000 units/ml.)	Local	2	3·0
	Remote	8	3·5
Chlortetracycline (0·025%) ..	Local	7	3·4
	Remote	8	2·8
Sulphadimidine (3%)	Local	7	3·1
	Remote	8	4·6
Chlorhexidine diacetate (0·1%)	Local	1	3·0
	Remote	10	2·3
Saline (controls)	Local	10	2·3
	Remote	10	2·1

All treatments given 20 minutes after infection to groups of 10 mice.

some antibiotics and a sulphonamide under the conditions described above.

It will be seen that the results with penicillin, chlortetracycline, and sulphadimidine were very similar whether these drugs were given locally or at a remote site. With streptomycin, local treatment was considerably more effective. This is in accordance with Garrod's (1948) demonstration that streptomycin is much more rapidly bactericidal than penicillin. The ineffectiveness of the single application of penicillin may be noted: Williams and Miles (1949) observed that a single application of penicillin to accidental wounds did not reduce the sepsis rate. Remote treatment with chlorhexidine diacetate was quite ineffective, as would be expected from its known lack of systemic activity (Davies, Francis, Martin, Rose, and Swain, 1954), but when applied locally it was highly effective.

The technique described above was devised during laboratory studies on chlorhexidine, many of which were carried out in order to compare the new antiseptic with older ones. Various salts of chlorhexidine have been examined over a range of concentrations on 28 occasions. These salts have included the relatively soluble (1.9%) diacetate, the less soluble hydrochloride (0.2%), and the very sparingly soluble (0.01%) carbonate, silicate, and metaphosphate. In direct comparison with one another all appeared to be equally effective. The results obtained are summarized in Table II, in which no distinction has been made

TABLE II

RESULTS OF TREATING STREPTOCOCCAL "WOUNDS" WITH CHLORHEXIDINE SALTS

Strength of Chlorhexidine Salt Used	No. of Mice Treated	No. of Mice Dead	Percentage Survivors
0 (controls)	280	280	0
0·1%	190	32	83
0·05%	50	9	82
0·02%	30	12	60
0·01%	10	1	90

between the various salts. It is clear that a highly significant effect was produced by the treatments.

The effect of increasing the interval between the deposition of organisms subcutaneously and the application of the antiseptic was studied. Seven groups of mice were anaesthetized and given the usual subcutaneous injection of diluted streptococcal culture. At intervals of 20 minutes, one hour, two, four, and six hours thereafter, groups were treated with chlorhexidine diacetate solution (0.1%) in the usual way, the original needle being left *in situ* and the mice maintained in a state of anaesthesia by occasional further intraperitoneal injections of tribromethanol. In this experiment, 90% horse blood was used as the vehicle for both the infecting organisms and the antiseptic (see below). Two control groups were included, one treated with saline in 90% blood after 20 minutes and one after six hours. The results are shown in Table III.

TABLE III

EFFECT OF DELAY IN TREATMENT OF STREPTOCOCCAL "WOUNDS"

Interval between Injection of Organisms and Treatment	Total No. of Deaths	Mean Survival Time of Mice Dying (Days)
20 minutes	3	4·3
1 hour	1	3·0
2 hours	1	2·0
4 ,,	4	3·6
6 ,,	6	2·6
Controls:		
20 minutes	10	3·0
6 hours	10	2·4

All "wounds" treated with chlorhexidine diacetate, 0·1% in 90% horse blood. Controls treated with saline in 90% horse blood. Groups of 10 mice.

The mean survival time of the mice which died was approximately the same in all groups, although more mice died in the groups treated after a long interval than in those treated promptly. This could have been due to gradual displacement of the indwelling needle. However, some mice were saved by the delayed treatments, indicating that organisms still accessible to the antiseptic were controlled by it.

The antiseptic powers of chlorhexidine under these experimental conditions have been compared with those of proflavine hemisulphate and *m*-chloroxylenol (Table IV), and also with those of various quaternary ammonium compounds (Table V). The experiment summarized in Table IV was carried out in two parts, in one of which

TABLE IV

TREATMENT OF STREPTOCOCCAL "WOUNDS" WITH CHLORHEXIDINE, PROFLAVINE HEMISULPHATE, AND *m*-CHLOROXYLENOL

Treatment	Vehicle	Total Deaths	Mean Survival Time of Mice Dying (Days)
Chlorhexidine diacetate, 0·05%	Saline	5	3·0
	Blood	3	2·8
Proflavine hemisulphate, 0·1%	Saline	6	2·7
	Blood	14	3·0
m-Chloroxylenol solution, 5%*	Saline	20	2·5
	Blood	20	1·7
None (controls)	—	20	1·7

Each group comprised 20 mice.

*Commercial solution containing 4·8% of *m*-chloroxylenol, i.e., treatment given contained 0·24% of antiseptic, the manufacturer's recommended concentration.

TABLE V

TREATMENT OF STREPTOCOCCAL "WOUNDS" WITH CHLORHEXIDINE AND QUATERNARY AMMONIUM COMPOUNDS

Treatment	Total Deaths	Mean Survival Time of Mice Dying (Days)
Chlorhexidine diacetate, 0·05%* ..	0	—
Cetrimide B.P., 0·5%*	8	3·0
,, ,, 0·1%*	10	2·4
Benzalkonium chloride B.P., 0·1%*	10	2·6
Domiphen bromide B.P.C., 0·5%*	9	2·7
Phenoctide, 0·5%*	10	1·6
None (controls)	10	2·1

Organisms and drugs were administered in 90% blood. Each group comprised 10 mice.

* Concentration recommended by the manufacturer for the treatment of wounds.

the vehicle for streptococci and antiseptics was saline and in the other was 90% horse blood.

From the results summarized in Tables IV and V it is clear that the marked effect of chlorhexidine under these conditions is unaffected by the presence of blood. There was a significant reduction of the action of proflavine when blood was present. The effects produced by the other antiseptics were very slight.

Experiments in which iodine, as a 0.5% solution in potassium iodide, the strongest which could be used, was the antiseptic, were carried out on six occasions in the presence and the absence of added blood. The results, which were very variable, are summarized in Table VI. There is no doubt that

TABLE VI

TREATMENT OF STREPTOCOCCAL "WOUNDS" WITH IODINE IN PRESENCE AND ABSENCE OF BLOOD

Treatment	No. of Mice Dead
0·5% iodine in water	30 out of 60
,, ,, ,, 90% blood	37 ,, ,, 39
None (controls)	60 ,, ,, 60

iodine is an efficient antiseptic for the destruction of organisms on the surface of the skin (Gardner, 1948), but its value as a disinfectant of wounds is unproved, as with all other disinfectants. The present tests showed a very marked reduction of activity in the presence of blood.

A number of substances which have been used as antiseptics for many years have been examined on various occasions. Without giving the results in detail, it may be said that mercuric oxycyanide (0.05%), mercuric biniodide (0.1%), 2:7-dibromo-4-hydroxymercurifluorescein (0.1%), 8-hydroxyquinoline (0.1%), potassium permanganate (0.1–1.0%), phenol (0.25–1.0%), and iodoform (1.0%) were without action, mercuric chloride (0.01%), sodium ethylmercuri-thiosalicylate (0.02–0.1%), and silver proteinate, Argyrol type (5%), showed a doubtful effect, whilst crystal violet (0.1%) and malachite green (0.1%) were slightly active. Acriflavine as a 0.1% aqueous solution was clearly active, but acriflavine emulsion B.P.C. was much less effective. The use of 4-aminomethylbenzene sulphonamide ("marfanil") (3%), used as a dusting powder for wounds by the German forces, was without effect.

Summary and Conclusions

The technique described has proved easy to carry out and has given reasonably reproducible results, although great precision cannot be claimed for it. The observations of Hewitt (1954) suggest that in the basic procedure described above the injected bacteria are surrounded by the subsequently injected antiseptic solution. It is difficult to imagine that a substance which is inactive under these rather favourable conditions could be very effective in clinical practice, but we cannot conclude that the converse is true. Results unduly favourable to any particular antiseptic used are not likely to be obtained, because the most probable error, accidental movement of the indwelling needle in the interval between infecting and treating the mice, would lead to misplacement of the antiseptic, with a consequent reduction of activity. This may, indeed, be the cause of the few early deaths often observed in groups treated with what appear to be, from the satisfactory condition of the bulk of mice in the group at the end of a week, effective treatments.

The technique appears capable of differentiating between compounds of marked inhibitory action *in vitro*, and also shows the marked reduction in activity *in vivo* which occurs with some substances when an excess of blood is introduced into the system. Chlorhexidine compares favourably with many other antiseptics under these experimental conditions.

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THE BIOCHEMISTRY AND ANTIBIOTIC SENSITIVITY OF THE *KLEBSIELLAE*

BY

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This paper presents a biochemical survey of a series of *Klebsiellae*. These findings are interpreted in the light of current taxonomical concepts. The antibiotic sensitivity of these organisms is also indicated.

Material

The material analysed here comprises a total of 82 strains. Thirty-two come from the National Collection of Type Cultures and are described in Table I. The remaining 50 strains were isolated at the Institute of Laryngology and Otology, mainly from infections of the upper respiratory tract. Data as to their source and serotype have already been presented (Epstein and Friedmann, 1958). In addition, the virulence of some of these strains for mice by oral and peritoneal routes of inoculation and the histopathology of pneumonic infections induced by them have been studied (Epstein and Payne, 1959 ; Epstein, 1959).

Techniques

Routine Biochemical Techniques.—All tests were performed at 37° C. and media inoculated by Pasteur pipettes from overnight peptone water growths. Cyanide and Koser citrate media were inoculated by loops and straight wires respectively. Unless otherwise stated, all tests were read daily for seven days.

Carbohydrate Fermentation. — Sixteen carbohydrates listed in Table II were tested with Andrade's indicator in media consisting of 1% carbohydrate (w/v) in peptone water.

Urease Test.—Christensen's (1946) weakly buffered urea agar medium was used.

Methyl-red and Voges-Proskauer Test.—The combined Ministry of Health (1939) oxoid media were used, and tests read at two days.

Koser Citrate Test.—Koser's (1923) medium was used, and positives confirmed by re-inoculation.

H_2S and Indole Production.—The combined SIM Difco (1953) medium was used and tested for indole at two and seven days.

Gelatin Liquefaction.—Liquefaction was observed in Difco gelatin agar stabs.

Nitrate Reduction.—Kauffmann's (1954) medium was used.

Cyanide Test.—The medium used was Taylor's (1956) modification of that described by Vagn Møller (1954b). This test is based on the finding by Braun

TABLE I

SOURCE AND SEROTYPE OF N.C.T.C. STRAINS ON *KLEBSIELLAE*

Strain	Type	Source
K. pneumoniae (12)		
5054	1	Pneumonia
9494	1	Sputum
9502	2	,,
9503	2	Urine
5055	2	Unknown
9128	8	,,
9129	9	,,
9130	10	,,
9131	11	,,
9132	12	,,
9133	13	,,
3279	Untyped	Pneumonia coyput
K. rhinoscleromatis (6)		
5046	3	Nose
5047	3	,,
5048	3	,,
5049	3 (acapsulate form 5048)	,,
1936	3	,,
7799	3	,,
K. ozaenae (7)		
5050	4	Nose
5051	5	Unknown
5053	5 (acapsulate form 5051)	,,
5052	6	,,
8883	Untyped	Sputum
9601	,,	Nose
8895	,,	Sputum
K. "*aerogenes*" (7)		
9527	I	Water
8844	II	Ice cream
8167	II	Water
8824	II	,,
8851	II	,,
8801	Untyped	Sputum
8808	,,	Water

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(1938) that the inhibitory effect of potassium cyanide on *Klebsiellae* is less than on *Escherichiae*.

Base.—The composition is as follows:

Evans' peptone	3 g.
NaCl	5 g.
KH_2PO_4	0.225 g.
$Na_2HPO_4.2H_2O$	5.6 g.
Aq. dist.	100 ml.

The base is adjusted to *p*H 7.6 and then autoclaved in flasks. To 100 ml. of base, 1.5 ml. of a freshly prepared 0.5% potassium cyanide solution in sterile distilled water is added, giving a concentration of 1/13,300. The medium is distributed in 1 ml. amounts in sterile bijou bottles and at 4° C. is stable for one month. Readings are made at 24 and 48 hours. False positives may be obtained unless the caps of bottles are screwed tightly to achieve partial anaerobiosis.

Gluconate Test.—The medium is a modification by Shaw and Clarke (1955) of that originally described by Haynes (1951) for the identification of Pseudomonads. The test is based on the ability of some organisms to metabolize glucose by direct oxidation instead of by the usual oxidative phosphorylation. Gluconate is oxidized to a reducing compound, presumed to be potassium-2-ketogluconate. The medium is tubed in 3 ml. amounts and tested at two days with Benedict's qualitative reagent.

Malonate and Phenylpyruvic Acid Tests.—Shaw and Clarke (1955) combined Leifson's (1933) sodium malonate and Henriksen's (1950) phenylalanine oxidase test in a single medium by adding phenylalanine to Leifson's medium and supplementing with yeastrel. Both tests were read at 24 hours.

Amino-acid Decarboxylases.—The technique described here is based on Shaw and Clarke's (1955) method. It was, however, found impossible to obtain homogeneous suspensions from some mucoid strains of *Klebsiellae* by washing off agar slopes, and for this reason suspensions were harvested instead from fluid media.

Reagents.—All seven L. amino-acids listed in Table III were tested at *p*H 5.0 and 0.03 M, except glutamic acid, in which case the concentration was reduced to 0.01 M because of its strong buffer action at a low *p*H. A 0.0125 M phthalate buffer at *p*H 5.0 was used. The indicator was 0.04% bromcresol purple in distilled water at *p*H 5.0. All reagents were stored at 4° C., with a drop of toluene added to prevent the growth of moulds.

Tubes.—Non-sterile 3 in. by ¼ in. tubes were used. Scrupulous chemical cleanliness was, however, essential, as traces of detergent produced false positive results.

Suspensions.—Strains under test were grown in peptone water at 37° C. for four hours.

As maximal decarboxylase activity develops at an acid *p*H, they were then subcultured to glucose broth and incubated for 16 hours at 37° C. The broths were then centrifuged at 3,000 r.p.m. for five minutes and supernatants discarded. The deposits were suspended in saline, centrifuged, and the supernatants again discarded. Approximately 0.5 ml. of distilled water at *p*H 5.0 was added, and the deposits resuspended to give suspensions of concentrations greater than 10^9 organisms/ml. (opacity tubes). As shown by Vagn Møller (1954a), the permeability of the bacterial cell membrane to amino-acids is increased by treatment with various lipoid solvents. Consequently 0.05 ml. of toluene was added to the suspensions, which were then incubated at 37° C. for 10 minutes.

Test.—Apart from controls, seven tubes corresponding to the substrates were required for each strain under test. To each tube the following were added with a 50 dropper: (1) 2 drops of suspension, (2) 2 drops of substrate, (3) 2 drops of buffer, (4) 1 drop of indicator. The mixtures were then incubated at 37° C. for four hours.

Controls.—Two controls were employed with each set of tests, namely, (1) buffer, suspension, and indicator (no substrate); (2) buffer, heat-killed suspension, indicator, and substrate.

Readings.—The *p*H change was read at half, one, and four hours. Vagn Møller (1955) indicated that under these conditions of test *p*H change in an arginine substrate can be affected by arginine dihydrolase besides decarboxylase. For this reason the specificity of *p*H changes in arginine tubes was checked by Nesslerization, as ammonia formation precludes significant decarboxylase activity. Random tubes producing an indicator change in the lysine substrate were checked chromatographically. Butanol with $2N.NH_4OH$ was the solvent and an ascending technique with Whatman No. 1 paper was used. Spots were identified with 0.1% ninhydrin in chloroform.

Amino-acid Oxidases.—The technique is similar to that described for decarboxylase determinations. These tests, however, were carried out at a more alkaline *p*H, and the production of a keto-acid determined colorimetrically with ferric chloride rather than by indicator change.

Reagents.—L. amino-acids at *p*H 6.8 of the same molarity as previously were used. The buffer was 0.025 M phosphate at *p*H 6.8, and the keto-acid reagent 0.5 M ferric chloride.

Suspensions.—These were similarly prepared, except that strains were grown in nutrient rather than glucose broth and subsequently suspended in distilled water at *p*H 6.8.

Tests.—The mixtures were incubated at 37° C. and samples tested at one and 24 hours with a drop of ferric chloride.

Antibiotic Sensitivity.—Sensitivity to the eight antibiotics in Table II was tested by Kohn's (1953a) simple diffusion technique. Sensitivity limits of organisms inhibited by erythromycin, oleandomycin, and novobiocin were estimated by the usual tube technique. Titrations extended over a range of 40 to 0.15 units.

Results

Table II summarizes in percentage form the results of the above tests. For purposes of convenience, the 82 analysed strains are arranged in the seven groups indicated. Whereas all untyped and acapsulate strains from the Institute of Laryngology and Otology and the untyped N.C.T.C. strain 3279 are relegated to their appropriate group, the two acapsulate N.C.T.C. strains are classified with their corresponding capsular types. The captions *rhinoscleroma*, *ozaenae*, and *aerogenes* are used without prejudice at this stage.

The decarboxylase activity of all strains is analysed in Table III. Under the conditions of test, a shift of *p*H after half an hour is indicative of feeble enzyme activity. Feeble arginine decarboxylase activity was demonstrated in 40 strains. The specificity of these delayed *p*H changes was confirmed by Nesslerization, with negative results in all cases. As is apparent, the majority of typed strains, including aerogenes, but with the notable exception of all but one type 3 and more than half type 4–6 strains, possessed strong lysine in decarboxylase activity. These results are also listed in Table II for purposes of correlation with the other tests. The reaction products of randomly selected strains, exhibiting strong lysine decarboxylase activity, were determined chromatographically and spots with R_f values equivalent to cadaverine (decarboxylated lysine) were obtained (Fig. 1). By use of the combined malonate and phenylpyruvic acid medium, phenylalanine oxidase activity was detected in only one type 1 strain. Micro tests, however, proved uniformly negative.

Antibiotic sensitivity results determined by a diffusion technique are listed in Table II. Titrations demonstrated that all anaerogenic *rhino-*

TABLE II
SUMMARY OF BIOCHEMISTRY AND ANTIBIOTIC SENSITIVITY OF 82 STRAINS OF *KLEBSIELLAE**

	K. pneumoniae Type 1	*K. pneumoniae* Type 2	*K. rhinoscleromatis* Type 3	*K. ozaenae* Types 4–6	*K. pneumoniae* Types 8–13	Untypable and Acapsulate Strains	*K. aerogenes*
No. of strains	23	5	7	9	6	25	7
Mucoid colonies	91	80	85	77	100	56	0
Tenacious colonies	78	60	71	66	16	4	0
Capsules	100	100	85	88	100	76	57
Motility	0	0	0	0	0	4	0
Adonitol	96	100	100	100	100	88	100
Inositol	74	80	42	100	100	52	100
Dulcitol	13	0	14	0	16	8	28
Glucose	100	100	100	100	100	100	100
,, gas	91	100	14	33	83	96	85
Lactose	96	100	14	66	100	100	100
,, gas	87	100	14	55	100	92	85
Saccharose	100	100	100	55	100	100	100
Mannitol	100	100	100	100	100	100	100
Maltose	100	100	100	88	100	100	100
Rhamnose	100	100	85	100	100	100	100
Xylose	100	100	100	100	100	100	100
Arabinose	100	100	100	100	100	100	100
Trehalose	100	100	100	100	100	100	100
Sorbitol	100	100	100	100	100	100	100
Salicin	96	100	100	100	100	96	100
Glycerol	61	100	85	100	100	52	100
Starch	100	100	100	100	100	100	100
Urea	87	100	14	44	100	52	85
Koser citrate	65	100	14	44	100	100	85
Voges Proskauer	87	60	0	0	50	96	57
Methyl red	13	40	100	100	50	4	42
Nitrate	96	100	100	88	100	100	100
Gelatin	0	0	0	0	0	0	0
Indole	0	0	0	0	0	8	71
H_2S	0	0	0	0	0	0	0
Cyanide	100	100	42	77	100	88	100
Gluconate	100	100	14	33	100	100	100
Malonate	47	100	71	11	66	100	85
Phenylpyruvic acid	4	0	0	0	0	0	0
Lysine decarboxylase	100	100	14	33	100	24	100
Percentage of strains sensitive to:							
Penicillin	0	0	0	0	0	0	0
Tetracycline	100	100	100	100	100	100	100
Streptomycin	100	100	100	100	100	96	100
Chloramphenicol	100	100	100	100	100	100	100
Polymyxin	78	60	71	88	66	60	71
Erythromycin	0	0	85	0	0	0	0
Oleandomycin	0	0	85	0	0	0	0
Novobiocin	0	0	85	0	0	0	0

*Expressed as percentages of strains giving positive reactions.

FIG. 1.—Chromatographic identification of cadaverine produced by enzymatic decarboxylation of lysine. (1) *Test: Klebsiella* suspension, buffer, indicator, and lysine. (2) *Control:* Buffer, indicator, and cadaverine. (3) *Control:* Heated *Klebsiella* suspension, buffer, indicator, and lysine. (Solvent—butanol with 2N. NH_4OH: Whatman No. 1 paper; time of running—16 hr.)

TABLE III
AMINO-ACID DECARBOXYLASE ACTIVITY IN *KLEBSIELLAE*

	No.	Lysine				Arginine		Ornithine		Histidine		Tyrosine		Glutamic Acid		Phenyl-alanine	
		−	±	+	++	−	±	−	±	−	±	−	±	−	±	−	±
Type 1	23				23	10	13	18	5	23		23		23		23	
,, 2	5				5	1	4	4	1	5		5		4	1	5	
,, 3	7	5	1		1	7		7		7		7		7		7	
Types 4–6	9	4	2		3	9		9		9		9		9		9	
,, 8–13	6				6	2	4	6		6		6		6		6	
Untypable and acapsulate ..	25	3	9		13	7	18	10	15	25		25		10	15	20	5
"Aerogenes"	7				7	6	1	7		7		7		7		7	
Total ..	82	12	12		58	42	40	61	21	82		82		66	16	77	5

++ = *p*H change less than ½ hour. + = *p*H change ½–1 hour. ± = *p*H change 1–4 hours. − = No change at 4 hours.

scleroma strains were sensitive to erythromycin, oleandomycin, and novobiocin over a range of 0.3 to 1.25 ml. with the exceptions of strain 1936 sensitive to 10 u./ml. oleandomycin and 5 u./ml. novobiocin, and strain 7799 sensitive to 5 u./ml. oleandomycin.

Discussion

Bergey's "Manual" (Breed, Murray, and Hitchens, 1948) divides the tribe *Escherichieae* into three genera, namely, *Escherichia, Aerobacter,* and *Klebsiella.* Edwards (1929) challenged the identity of *Aerobacter,* of which *aerogenes* was the type species, and concluded that there was no justification for its extrageneric differentiation from *Klebsiellae.* Kauffmann (1949), among other workers, proposed that *aerogenes* strains be classified in the *Klebsiella* genus. Nearly all strains originally submitted to the N.C.T.C. as *aerogenes* have recently been reclassified as *K. pneumoniae* (Medical Research Council, 1958). Cowan (1956) in a recent survey of problems of nomenclature suggested that *aerogenes* be accepted as a *Klebsiella* subgroup for the convenience of water bacteriologists, who would be loath to report on the isolation of *K. pneumoniae* from a potable water. By implication, however, he accepted that source of origin is not a valid taxonomic criterion and that the designation *aerogenes* has thus no true status. Furthermore, Clarke and Tracey (1956) recently ascertained that high yields of chitinase and cellulase, constitutive enzymes supposedly characterizing many species of soil and water bacteria including *aerogenes,* can also be isolated from *Klebsiellae* of human origin. Cowan (1954) also indicated the undesirability of the epithet *pneumoniae* for the *Klebsiella* type species. He suggested instead the name *friedlaenderi,* which does not suggest an obligate relationship with respiratory infections.

The *Klebsiella* genus was originally intended to include a heterogeneous group of non-motile capsulated organisms, with an indole, methyl red, Voges-Proskauer and citrate formula (1MViC) −−++. Both intrageneric and extrageneric biochemical and serological overlap have complicated the issue. Whether or not a particular organism be relegated to this genus may depend on the relative emphasis placed on any particular criterion. Serologically, for instance, an organism may belong to the *Escherichiae* and biochemically to the *Klebsiellae*—or vice versa. In such cases a decision as to which property represents the aberrance may prove impossible. In an attempt to discourage undue reliance on any given criterion as a basis for classification, Clarke (1955) suggested that the distribution of biochemical characters be best described on a statistical rather than on an "all or none" basis. Sneath (1957) has recently lent practical emphasis to this concept by demonstrating the application of mathematical computers to such taxonomical problems.

Kauffmann (1949, 1954) accepted only two biochemical species, *K. pneumoniae* and *rhinoscleroma.* Edwards and Fife (1955) further stressed that types 4–6, sometimes associated with *ozaena,* represent a diffuse biochemical transition between these two species and not a discrete entity. Henriksen (1952) proposed that *ozaenae* and *aerogenes* species be also recognized. Other workers, including Ørskov (1957), recommended the acceptance of further biochemical subtypes. The perpetuation of the name "*rhinoscleroma*" for type 3 organisms is based on tenuous evidence. Occasionally type 3 strains can be isolated from extranasal sites. Moreover, an aetiological relationship between the organism and disease is not proven. It is felt consequently that such strains should preferably be described numerically by their capsular antigen. The status of motile strains is still *sub judice.* Those liquefying gelatin are best classified as *Cloaca cloacae,* in accordance with Kauffmann (1954), or, as Cowan (1956) suggests, *Klebsiella cloacae.* Edwards and Fife

(1955) recommend the designation *Aerobacter cloacae*. Possibly non-liquefying strains represent biochemical variants, and, as Cowan (1956) further proposes, should also be included. The Institute of Laryngology and Otology strain 3502 of the present series was such a motile non-liquefying strain.

Typical *pneumoniae* strains are usually aerogenic, lactose, citrate, Voges-Proskauer, malonate, cyanide, gluconate, and lysine positive. Type 3 strains possess the reverse properties. Strains belonging to types 4–6 are sometimes aerogenic, citrate, cyanide, and lysine positive, and usually malonate and Voges-Proskauer negative. However, as indicated in Table II, biochemical aberrance is common in *pneumoniae*, especially in type 1 strains. It is less frequent in type 3 strains. One of the seven type 3 strains of the present series was aerogenic, lactose, and lysine positive. In fact, although serologically this strain (No. 615) belonged to type 3, it exhibited the biochemical features of a typical *pneumoniae*. Henriksen (1954) also described such strains and discussed this problem of mutually exclusive criteria.

Of the 82 strains of *Klebsiellae* studied in the present series, 15 were anaerogenic, of which six belonged to type 3 and five to types 4–6. As indicated previously, gas production in type 3 strains can be correlated with other biochemical features. The majority of anaerogenic strains were Voges-Proskauer negative and failed to ferment lactose. There were no other lactose-negative strains. Pickett and Cabelli (1953) stressed the low degree of biochemical reactivity of their lactose-negative strains, the majority of which, unlike those studied here, also failed to ferment adonitol and inositol. The fermentations of the former and to a lesser extent of the latter are fairly reliable differential tests between *Klebsiellae* and *Escherichiae*. Occasional strains failing to ferment these carbohydrates appeared otherwise biochemically typical. Dulcitol fermentation appears rare in *Klebsiellae*. Kauffmann (1954) pointed out that type 10 is characteristically dulcitol positive and this was in fact confirmed by the behaviour of the single type 10 strain studied. In addition, the aerogenic type 3 strain was also positive. In the few remaining dulcitol-positive strains, no other constant or significant biochemical deviation was observed.

Kligler (1914), on the basis of a biochemical comparison between *cloacae* and *aerogenes* strains, maintained that gelatin liquefaction was inversely related to gas production from glycerol. Edwards and Fife (1955) have shown, however, that *Klebsiellae* occasionally fail to produce gas from glycerol, as did eight strains in the present series. No gelatin-liquefying strains were isolated, although, as Lautrop (1956) pointed out, extended incubation or the use of the Kohn (1953b) technique may possibly unmask some liquefiers. Delayed hydrolysis of urea and utilization of citrate is characteristic of most *pneumoniae* strains. Type 3 strains, with the exception of the aerogenic variant, were uniformly negative. Type 4–6 strains again behaved in an intermediate fashion, and those utilizing citrate did so only after four days. Occasional *pneumoniae* strains were also citrate and urease negative. Kauffmann (1954) pointed out that citrate-positive *Escherichiae* are very rare, and that no urease-positive strains have ever been described. Cowan (1957) has, however, encountered urease positive *Escherichiae*. Contrary to the findings of Kauffmann (1954), the single strains of *Klebsiellae* types 9 and 10 studied in this series were citrate positive. The methyl red and Voges-Proskauer are by no means clear-cut tests. Results depend on temperature, duration of incubation, method of testing, and, as indicated by Jennens (1954), the nature of the substrate. Of the 89 strains of *Klebsiella pneumoniae* recently studied by Vagn Møller (1954c), 89% were Voges-Proskauer positive at one day, but only 45% at four days. Variables such as these make it difficult to compare results under different conditions of test. However, in this study all type 3–6 strains were methyl red positive. Contrary to Leifson (1933), who found an absolute correlation between Voges-Proskauer production and malonate utilization, the strains studied here exhibited an approximate correlation only. Indole production, while rare in *Klebsiellae*, is usual in *Escherichiae*. Of 626 *Klebsiella* strains studied by Edwards and Fife (1955), only 26 were indole positive. "*Aerogenes*" type II strains are rare, but indole positive by definition. Apart from these, only two other strains from the Institute of Laryngology and Otology produced indole, namely, the motile strain 3502 referred to previously and also the acapsulate strain 3872 which was otherwise biochemically typical. Reduction of nitrates is a non-specific test and only one negative was encountered. It is generally accepted that failure to produce H_2S is a reliable exclusion test for *Klebsiellae*; all strains in this series were negative. Clarke (1953) has, however, demonstrated that by the use of N.C.T.C. methods of testing the reverse findings may be elicited.

Resistance to cyanide inhibition and the ability to form reducing end-products from gluconate

are differential tests of the utmost value. With the exception of some type 3–6 strains, the great majority of *Klebsiellae* tested were positive. There was, moreover, almost an absolute correlation between these tests.

The classical manometric studies of Gale (1946) on the decarboxylases demonstrated the varied and distinctive pattern of the enzymatic repertoire of different groups of bacteria. Subsequent workers, notably Woiwod (1949), Vagn Møller (1954c, 1955), and Shaw and Clarke (1955), introduced a variety of simplified techniques and stressed their practical application. Vagn Møller (1954c) examined a total of 671 *Enterobacteriaceae* and found that no strain, apart from *K. pneumoniae*, possessed powerful lysine and no other decarboxylase. The specificity of this test, as confirmed by the results in the present series, is thus high. Anaerogenic type 3 strains are consistently negative and *pneumoniae* strains almost invariably positive. Type 4–6 strains behaved in a variable manner, as did untyped and acapsulate strains. Unlike Shaw and Clarke (1955), who were able to demonstrate weak phenylalanine oxidase activity in some *Klebsiellae*, this was detected in one strain only by the combined medium and not by the micro test.

All strains examined were penicillin resistant, tetracycline, chloramphenicol, and streptomycin sensitive, with the exception of one, resistant to the last antibiotic. There is ample evidence, as reported by Finland, Murray, Harris, Kilham, and Meads (1946) and Ørskov (1952), that resistance to streptomycin often develops during treatment. Approximately two-thirds of strains were polymyxin sensitive. There appear to be scanty data on the sensitivity of *Klebsiellae* to some of the more recently introduced antibiotics. Coppo (1957) found that the minimum inhibitory concentration of novobiocin against 10 strains of "*aerogenes*" ranged from 250 to 500 u./ml. Erasmus (1956) reported on seven erythromycin-resistant untyped *Klebsiellae*. Apart from the aerogenic variant, all other type 3 strains exhibited suprising sensitivity to erythromycin, oleandomycin, and novobiocin. It is interesting that this previously unrecorded sensitivity pattern is restricted to the anaerogenic type 3 strains, which, as suggested previously, represent a homogeneous biochemical subtype.

Summary

A biochemical analysis of 82 *Klebsiella* strains is reported and some problems of nomenclature discussed. It is recommended that the name *friedlaenderi* be substituted for *pneumoniae* as the *Klebsiella* type species and further that type 3 strains be no longer designated *rhinoscleroma*. No evidence for any other valid species was found.

It is suggested that the distribution of various properties, including biochemical, in the *Klebsiella* genus be best described on a statistical rather than on an "all or none" basis. It is further emphasized that the results of any biochemical test must be qualified by reference to the particular technique employed. Biochemical aberrance is common in *pneumoniae*, especially type 1 strains. *K. pneumoniae* is much more heterogeneous than the type 3 species, of which only two subtypes, the aerogenic and anaerogenic, appear to exist. Typical *pneumoniae* strains are usually aerogenic, lactose, citrate, Voges-Proskauer, malonate, cyanide, gluconate, and lysine positive, and exhibit delayed hydrolysis of urea. Type 3 strains, apart from the rare anaerogenic variety, possess the reverse properties. Type 4–6 strains on the whole behave in an intermediate fashion. The value of the cyanide, gluconate, and lysine decarboxylase tests is stressed, and there is a high degree of correlation between them. The majority of all *Klebsiella* strains ferment adonitol and inositol, but not dulcitol, fail to produce indole and H_2S, or to liquefy gelatin.

Attention is drawn to the sensitivity of anaerogenic type 3 strains to erythromycin, oleandomycin, and novobiocin. All other *Klebsiellae* are resistant to these antibiotics.

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LABORATORY INFECTION WITH *TOXOPLASMA*

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The main object of this paper is, by citing cases of laboratory infection with *Toxoplasma*, including one personal and hitherto unpublished, to point out the serious risks involved in working with this parasite and to stress the need for care. Another object is to see what can be learnt from these regrettable accidental human experiments about the signs and symptoms to be expected in toxoplasmosis.

Personal Case Report

In October, 1954, the writer started working with *Toxoplasma* in Bombay, and it involved the maintenance of the virulent RH strain by routine passage in mice, the inoculation of fertile eggs, and the performance of the dye test for *Toxoplasma* antibodies (Sabin and Feldman, 1948). No particular accident, such as a needle prick or a splash, is remembered, but it is likely that at times *Toxoplasma* got on to the skin, particularly in the performance of the dye test.

Before the work was started the serum was found by the dye test to be free from antibodies to *Toxoplasma*. It remained so till July 22, 1955. Thereafter it was not tested till the writer fell ill.

About the middle of November, 1955, the patient felt sick and depressed. Afterwards he experienced severe frontal headache accompanied by malaise and muscular pain. On November 18 his temperature rose to 103° F. and remained elevated for eight days. On the fourth day after the onset of the fever glands in the cervical, axillary, and inguinal regions enlarged and were tender to pressure.

Because of the nature of his work and the signs and symptoms of his illness, toxoplasmosis was suspected and the appropriate serological tests carried out with positive results as shown in Table I.

TABLE I

RESULTS OF SEROLOGICAL TESTS

Date	Dye Test	Complement Fixation Test
July, 1955 (3½ months before illness)	< 1: 4	< 1: 4
Jan., 1956 (2 months after onset)	1: 256	1: 8
Mar., 1956 (4 ,, ,, ,,)	Between 1: 256 and 1: 512	1: 16
Jan., 1957 (14 ,, ,, ,,)	1: 170	< 1: 4
April, 1957 (17 ,, ,, ,,)	1: 271	1: 20
May, 1957 (18 ,, ,, ,,)	1: 600	1: 40

Four months after the onset of illness the patient was given an 11-day course of treatment with 12.5 mg. "daraprim" daily and 6 g. sulphadiazine on the first day, followed by a daily dose of 4 g. Within six days of the start of the treatment the headache, which had persisted intermittently, and malaise disappeared. The glands remained enlarged and were, indeed, still enlarged in August, 1957.

Discussion

From the frequency with which *Toxoplasma* antibodies are found in the general population it is obvious that man can be readily infected, though strangely little is known of how this comes about. It is, therefore, not surprising that people who handle toxoplasms in the laboratory sometimes develop toxoplasmosis. Indeed, it is strange that this does not happen even more frequently. Perhaps the explanation may be that many people already have antibodies to *Toxoplasma*, due to natural subclinical infection, before they start to work with the organism. It is certainly striking that of the laboratory workers, whose cases are summarized in Table II, all except two were known to have been previously devoid of antibody. In one patient only (Case 17) was antibody known to have been previously present (dye test 1:64). This patient, in contrast to patient 10 who also squirted *Toxoplasma*-containing peritoneal exudate into the eye, had only a local manifestation, conjunctivitis. The dye test titre fell from 1:256 to the previous level of 1:64 in a very short period of three weeks. In most cases antibodies rose to high titres. In six of the patients they accompanied the isolation of the parasite and point to the specificity of the antibody tests. Of course, it does not follow that infection will lead to illness. H. E. (Case 6) developed high titre antibodies after he was bitten by an infected rabbit, but he was not ill. A. A. van S. (Case 18) had enlarged glands from which toxoplasms were isolated, but was afebrile and did not feel ill. E. H. (Case 14) had tonsillitis and salpingitis, but these were regarded as coincidental. There was only

TABLE II

Case No.	Reported by	Initials of Patient	Year of Infection	Incubation Period	Duration of Illness	Probable Cause of Infection	Headache	Fever	Lymph-adenopathy	Rash	Ocular Involvement	Respiratory Involvement	Myocarditis	Encephalitis and Other C.N.S. Disturbances	Highest Dye Test Titre	Highest C.F.T. Titre	Isolation of *Toxoplasma*
1	Ström (1951)	A.N.S.	1949	3 days	1 month	Needle prick	0	+	+	0	0	0	0	0	1:2,000	..	Not attempted
2	Ström (1951)	I.O.	1949	Unknown	At least 6 months	Worked with *Toxoplasma* for 18 days	+	+	+	+	Conjunctivitis	0	+	Meningoencephalitis	1:4,000	..	Unsuccessful. C.S.F., sputum, gland
3	Sexton *et al.* (1953)	V.F.	1951	,,	10 days	Unknown, worked with *Toxoplasma* for 2 years	..	+	0	+	Conjunctivitis	Moist rales	+	Irrational convulsions	1:256	..	From blood, C.S.F., brain, spleen, and liver
4	Brown and Jacobs (1956)	L.J.	1951	,,	2 months	Worked with *Toxoplasma* for 4 years. Splash in the face	+	+	+	+	0	0	0	0	1:4,096	1:80	Unsuccessful. Muscle biopsy and bone marrow
5	Wettingfield *et al.* (1956)	..	1954	,,	1 month	Unknown, worked with *Toxoplasma* for a year	+	+	+	+	0	Cough	0	0	1:1,000	++	From inguinal lymph node and blood
6	Sabin *et al.* (1952)	H.E.	1949	Asymptomatic		Rabbit bite	0	0	0	0	0	0	0	0	1:16,000	1:125	Not attempted
7	Alm (1949)	..	1949	Unknown	Several days	Worked with *Toxoplasma* for 1 month	..	..	..	..	..	..	..	..	1:4,000		Unsuccessful. Blood to chick embryos
8	Beverley *et al.* (1955)	S.C.M.	1953	9 days	3 weeks	Needle prick	+	+	+	0	0	0	0	0	1:4,200	1:16	Not attempted
9	Sabin (1950)	O.H. Prague	1950	Unknown	1 month	Unknown	..	..	..	..	..	..	..	..	..	..	From blood
10	Hormann (1955)	W.M.	1953	3 days till conjunctivitis, 10 days till generalized illness	Discharged from hospital on 52nd day. Enlarged glands for months after	Splash on face and eye	+	+	+	+	Swelling of eyelid, conjunctivitis	Bronchopneumonia	0	Meningoencephalitis	1:1,000	++	Not attempted
11	Mohr (1953)	Case 1	..	Unknown	2 weeks followed by relapse after 2 months	Unknown, worked with *Toxoplasma* for 4 weeks	+	..	..	..	..	..	..	..	1:100	++ Westphal	,, ,,
12	Mohr (1953)	,, 2	..	,,	Unknown, doubtful. relapse after 3 months	Unknown	+	+	..	..	..	Pneumothorax	..	..	Positive	..	,, ,,
13	Mohr (1953)	,, 3	..	,,	Unknown, relapse after 3 months	Unknown, worked with *Toxoplasma* for 3 weeks	+	+	..	..	..	..	..	..	1:200	..	,, ,,
14	Thalhammer (1954)	E.H.	1953	Asymptomatic		Unknown, worked with *Toxoplasma* for 2 years	..	..	..	..	..	Coincidental tonsillitis	..	..	1:262,334	..	,, ,,
15	Rawal (1959)	B.D.	1955	Unknown	4 months	Worked with *Toxoplasma* for 1 year	+	+	+	0	0	0	0	0	1:600	1:40	,, ,,
16	Wright (1957)	..	1955	9 days	28 days	Needle prick	..	+	+	0		Pneumonitis	0	0	1:4,096	1:32	From blood, lymph node
17	Straub (1955)	K.	..	3 ,,	5 ,,	Splash in both eyes	..	..	..	..	Swelling of eyelids, conjunctivitis		0	0	1:256	..	Not attempted
18	van Soestbergen (1957)	A. A. van S.	1952	8 ,,	Never really ill. Enlarged glands for several weeks	Needle prick	0	0	+	+	0	0	0	0	1:1,000	1:128	From subclavicular gland

one fatality in the reported cases (V. F., Case 3). In the other cases the duration of the illness varied from seven days to four months.

The commonest signs and symptoms were headache and malaise (nine cases) and lymphadenopathy (eight cases). In six cases a rash was reported. Three had encephalitic signs and two myocarditis. Two patients, in addition to the two who squirted *Toxoplasma*-containing material in their eyes, developed conjunctivitis.

In many cases the mode of infection was unknown. Four, however (Cases 1, 8, 16, and 18), were due to a needle prick. Cases 10 and 17 were due to a splash on the face and Case 4 may well have been due to a similar cause. Case 2 was thought to have contracted the illness in pipetting.

The cases in which the actual date of infection was known afford an excellent opportunity of assessing the incubation period. This varied from three days (two cases of splashes in the eye) to three to nine days (needle pricks).

Following the indications given by the therapeutic trials on experimental animals of Sabin and Warren (1942) six of the patients (Cases 1, 2, 3, 8, 10, 12) were treated with sulphonamides. Later patients (Cases 5, 15, 16) were given a combination of a sulphonamide and "daraprim" as first suggested by the work of Eyles and Coleman (1955). There are not enough cases to assess the value of therapy. All that can be said is that Case 8 was free from fever five days after the start of sulphonamide treatment; Case 3, on the other hand, died in spite of it. Cases 5 and 15 improved after treatment with sulphonamide and "daraprim." Case 16 was free of fever 36 hours after the start of treatment. Cases 11 and 13 were treated with aureomycin. Eyles (1956) reports that enormous doses of this drug, much larger than can be attained in man, are required to produce a favourable result in infected mice. It was used in addition to sulphadiazine in the patient in Case 3, who died, and also unsuccessfully in the case reported by Kass, Andrus, Adams, Turner, and Feldman (1952).

Three morals can be drawn. First, as with all dangerous organisms, great care should be taken to avoid contamination. A particular risk, and one likely to increase as more laboratories do it, is found in the performance of the dye test (Sabin and Feldman, 1948). Second, as far as possible, only persons who already have *Toxoplasma* antibodies should be employed on this work. Third, attention should be directed to the development of an effective vaccine for the protection of laboratory workers.

Summary

The writer reports the course of his own laboratory infection with *Toxoplasma* and reviews 17 others.

Headache, malaise, and lymphadenopathy were the commonest symptoms and signs.

The need for care when working with *Toxoplasma* is stressed.

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J. clin. Path. (1959), **12**, 62.

CONGENITAL PULMONARY LYMPHANGIECTASIS

BY

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An analysis of the literature reveals scanty data on congenital pulmonary lymphangiectasis (Virchow, 1856; Klebs, 1889; Bredt, 1952). It would therefore appear to be a comparatively rare condition, yet an investigation of just over 451 consecutive necropsies on stillbirths and neonates, carried out in the Portsmouth area, revealed five cases. Reports of three of these have already been published (Laurence, 1955); the other two are Cases 4 and 5 of the present communication. Case 6 was examined at The Hospital for Sick Children, Great Ormond Street, Cases 7–9 were referred from Birmingham, and Case 10 from Toronto. Additional examples have been reported by Giammalvo (1955) and by Maidman and Barnett (1957). It therefore appears that the condition is not rare, but unrecognized, especially in its microscopic form.

Case Reports

Cases 1–3.—These have already been reported by Laurence (1955).

Case 4.—A male infant was born spontaneously at term, limp and blue, after a normal pregnancy. The cyanosis continued though he became more energetic. On the fourth day congenital heart disease was suspected, possibly a left-to-right shunt, as a soft apical systolic murmur could be heard. A radiograph of the chest (Fig. 1) was taken, which showed an enlarged heart and increased opacity of both lung fields with some mottling, suggestive of a diffuse cystic change. The child was placed in an oxygen tent but without any effect on the cyanosis. By the sixth day the liver had enlarged, and there was a gradual fall in temperature, with a concomitant rise in the pulse and respiration rates. His condition deteriorated. On the eighth day he died.

At necropsy the infant (weight 3.1 kg.) was well formed, but markedly cyanosed. Both lungs were considerably heavier than normal, and studded with small, fluid-containing vesicles throughout; some of those on the surface resembled very dilated lymphatic vessels. The parenchyma itself was congested and consolidated, and in places mucopurulent material could be expressed from the air passages. The hilar lymph nodes were normal and the pleural cavities dry. The heart was somewhat dilated, but otherwise the remaining systems showed no abnormalities to the naked eye.

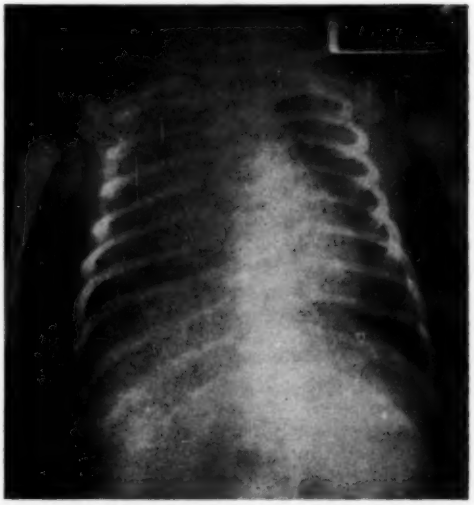

FIG. 1.—A radiograph of Case 4 showing diffuse opacity and mottling of both lung fields, suggestive of a generalized cystic condition.

Case 5.—A male infant, who progressed moderately well for a few days following a normal pregnancy and delivery, developed increasing cyanosis and vomited his feeds. Antibiotics settled the slight pyrexia (99.6° F.), but failed to improve the child's general condition. In spite of continuous oxygen the cyanosis increased. On the fifteenth day there was obvious congestive cardiac failure, which did not respond to digoxin, and he died on the thirtieth day of life.

At necropsy the infant, who weighed 3½ kg., was seen to be poorly nourished and markedly cyanosed.

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Bilateral serous pleural effusions were present (150 ml. in all). The lungs were abnormally firm and the subpleural lymphatics were prominent. The cut surface was pink and exuded abundant clear fluid. The gross appearances were thought to be those of pulmonary oedema and diffuse fibrosis. The heart (45 g.) showed moderate right ventricular dilatation and hypertrophy. The ductus arteriosus was only patent to a probe. A small serous pericardial effusion was present and the peritoneal cavity contained 200 ml. of ascitic fluid. The liver and spleen were large and congested.

Case 6.—This full-term male infant, in whom irregular heart sounds and meconium-stained liquor, suggesting foetal distress, were noted before breech delivery, was stillborn and the placenta was normal.

At necropsy at The Hospital for Sick Children the infant, who weighed only 2 lb. 10 oz., showed abnormalities of the eyes and arms and had a bilateral harelip and cleft palate.

Both lungs were hypoplastic and showed an exaggerated but fine lobular pattern with dilated subpleural lymphatic vessels, giving an appearance not unlike that of a liver with biliary cirrhosis. On cross-section, the parenchyma was unexpanded and divided into small lobules by numerous, irregular cysts measuring up to 1 mm. in diameter.

In addition there were hypoplastic kidneys, a tentorial tear, a left diaphragmatic hernia, and multiple malformations of the heart. These included a mitral atresia, a ventricular septal defect, a hypoplastic left ventricle, and a bicuspid pulmonary valve.

A full-term, stillborn, female sib also had a harelip and cleft palate and weighed 4 lb. 10 oz. Foetal distress was noted for which no cause was found at necropsy. Unfortunately, no sections are available for examination. From the description, however, it might be possible that this case was also an example of pulmonary lymphangiectasis.

Case 7.—A female infant was born spontaneously after a normal pregnancy, but with the cord wound tightly round her neck. She had "white asphyxia," gave one long gasp and died without respiration ever having been established.

At necropsy the infant weighed 2.1 kg. "The bulky unexpanded lungs showed a network of very prominent lymphatic vessels under the pleural surfaces. In many areas, especially near the base and the free borders, numerous subpleural cysts containing clear, serous fluid were found. On the cut surface the parenchyma presented a spongy appearance with fluid-containing cysts extending throughout the lung, dividing the parenchyma into very distinct lobules" (Dr. H. Baar). The pleural cavity and hilar lymph nodes were normal.

The heart was markedly enlarged and showed aortic stenosis with patent intraventricular and interauricular septae and hypoplasia of the left ventricle and mitral valve. The ductus arteriosus was widely dilated. Apart from a tentorial tear and a subdural haematoma, there were no other significant findings.

Case 8.—A male infant was born spontaneously after a normal pregnancy. He immediately became deeply cyanosed and hypotonic, and, in spite of mucus aspiration, died six hours after delivery.

At necropsy he was seen to be a normally developed but markedly cyanosed child (weight 2.2 kg.). "The lungs were bulky and greyish-white with innumerable pinhead-sized subpleural cysts scattered all over the surface. Some of the cysts were collapsed, but the majority were filled with clear, serous fluid. The lungs were fairly well aerated, and fluid-filled cysts, which were irregular in shape and size (up to 2 mm. in diameter), could be seen with the naked eye throughout the parenchyma. They tended to give the lung an exaggerated lobular pattern" (Dr. H. Baar). The pleural cavities and tracheo-bronchial lymph nodes were normal.

There was marked enlargement of the heart, chiefly of the right atrium and ventricle. The left ventricle and atrium and aortic ostium were hypoplastic. A large auricular septal defect and patent ductus arteriosus were present. The only other positive finding was a large right-sided subdural haematoma.

Case 9.—A male infant was born spontaneously, weighing 2.3 kg. Meconium-stained liquor was seen before the head was fully crowned, though there had been no previous signs of foetal distress. The child cried well, but his breathing was irregular. Cyanosis became marked in spite of the usual resuscitative measures and continuous oxygen, and he died two hours after delivery.

At necropsy the lungs were "voluminous and extremely oedematous. To the naked eye it seemed as if a thin layer of ice enveloped the whole lung immediately underneath the pleura. Through this layer, the wide lung markings were obvious" (Dr. H. Baar). The pleural cavities each contained 10 ml. of clear, serous fluid. Apart from the congestion of the cerebral vessels, and a small rupture of the great vein of Galen, there were no other significant findings.

Case 10.—A full-term male infant became cyanosed and flaccid 10 minutes after an uneventful delivery. He was transferred within an hour to hospital, but was dead on arrival.

At necropsy 15 hours later, deep cyanosis and petechial haemorrhages of the skin were found. Venous congestion was generalized, and particularly severe in the lungs. Bilateral subpleural haemorrhages were present, but no other abnormal features were noted on naked-eye examination of the lungs.

The liver was also markedly congested, and there was a haemorrhage into the left adrenal gland. A small left tentorial tear was present.

Gross Appearances of the Lungs

Many features common to all or some of the cases are at once apparent. The lungs are usually bulky and inelastic, with pronounced lobulation. Prominent

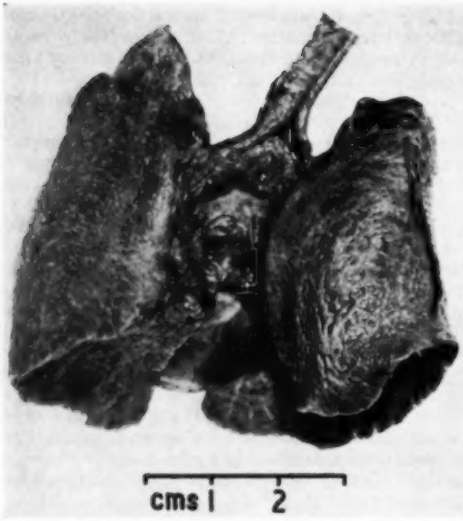

FIG. 2.—The lungs of Case 8 showing very prominent subpleural lymphatic vessels and subpleural cysts.

FIG. 3.—The lungs of Case 7 with exaggerated lobular pattern and subpleural cysts, some of which are collapsed and appear as dark depressions under the surface.

subpleural lymphatic vessels form a dense network (Fig. 2), and in the majority of cases subpleural cysts can be seen with the naked eye. These may measure up to 3 mm. in diameter, and are transparent and glistening. They are filled with clear, colourless serous fluid, and their thin walls have a smooth inner surface. After fixation, these cysts tend to collapse, and they are then seen as darkish depressions under the pleura (Fig. 3).

Section of the lung of a typical case shows a parenchyma diffusely honeycombed with fluid-containing cysts of irregular shape and size, some of which may reach as much as 5 mm. in diameter (Fig. 4). The cysts near the hilum are elongated and often enlarged (Fig. 5). In addition there is a considerable increase in the fibrous tissue of the interlobular septa, which, together with the cystic spaces, gives the lungs their grossly exaggerated lobular pattern. In all the cases studied, both lungs were almost uniformly affected by the cystic changes.

Serous fluid, suggestive of pulmonary oedema, may ooze from the cysts when the fresh lung is cut, while the abundant interlobular fibrous tissue may simulate the appearance of pulmonary fibrosis. Indeed, these diagnoses were made in three cases on gross examination (Cases 1, 5, and 9), and one was mistaken for interstitial emphysema (Case 3). The condition was, however, correctly recognized in four others (Cases 4, 6, 7, and 8), whereas the abnormality passed unnoticed in only two cases (Cases 2 and 10) until sections of the lungs were examined microscopically.

Histology

The cysts are situated mostly in the connective tissue under the pleura and in the interlobular septa (Figs. 6 and 7), though collections of smaller spaces can be found within the lobules themselves. They are often closely related to bronchi and blood vessels, sometimes sharing a common wall (Fig. 8).

The cyst walls, rarely more than 10 μ in thickness, consist of a delicate network of elastic, collagen, and occasionally smooth muscle fibres (Fig. 9). They are lined by a single layer of flat endothelial cells, which in Case 2 is extensively desquamated and curled up in the centre of the space.

Faintly eosinophilic material or red blood cells may be present in the spaces, but more usually these appear completely empty. In Case 4, however, many cavities contain pus cells, infection presumably having spread from the neighbouring air passages and alveoli (Fig. 10).

The spatial arrangement of the cystic spaces within the lung was investigated. Serial sections, 10 μ thick, were cut and the preparations projected on to cellulose acetate sheeting by an Aldis projector fitted with a microscope attachment; the

FIG. 4.—The lung of Case 7 cut at right angles to the main vessels and bronchi. The cysts show as dark rounded spaces.

outlines of the spaces were drawn in Indian ink in consecutive sections in one area, and in every fifth section in other but larger fields. The tracings were superimposed accurately one upon another after lightly colouring the "lumina" (Fig. 11). From these it became evident that the spaces are part of an intricate network of intercommunicating channels. The individual channels themselves vary in width, not only from one another, but also in different parts of the same channel. Thus, a space only 15 μ in diameter may be in direct continuity with a cystic dilatation 2 mm. in diameter. Further, the channels are constantly changing direction, and may double back upon themselves or take a spiral course. In the sections examined, none of the cysts appears to be without a communication with the remainder of the network, and so far no true valves have been found. Structures described as valves by Giammalvo (1955) and Maidman and Barnett (1957) were probably only folds in the channel wall.

The abundant connective tissue of the interlobular septa appears almost embryonic, and none of the cases shows thick collagen fibres of chronic inflammatory changes. In some instances, the lung parenchyma itself looks immature, resembling that of a seven-month foetus. Sometimes it is unexpanded, sometimes partially or well aerated, and in one case there is a terminal pneumonia. Alveoli may contain red blood cells

FIG. 5.—Lung of Case 7 cut in the plane of the main vessels and bronchi. The cysts appear as dark spaces which tend to be elongated towards the hilum.

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FIG. 6.—Lung of Case 2 to show distribution of lymphatic spaces and abundant interlobular connective tissue (haematoxylin and eosin × 16).

FIG. 7.—Lung of Case 7 showing distribution of cysts in close relationship to blood vessels (haematoxylin and eosin × 10).

FIG. 8.—Lung of Case 10 with subpleural spaces in an interlobular septum in close relationship to blood vessels (haematoxylin and eosin × 35).

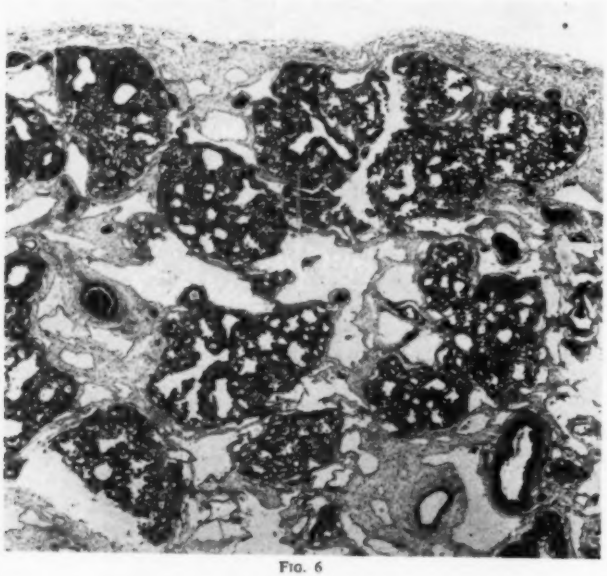

FIG. 6

FIG. 8

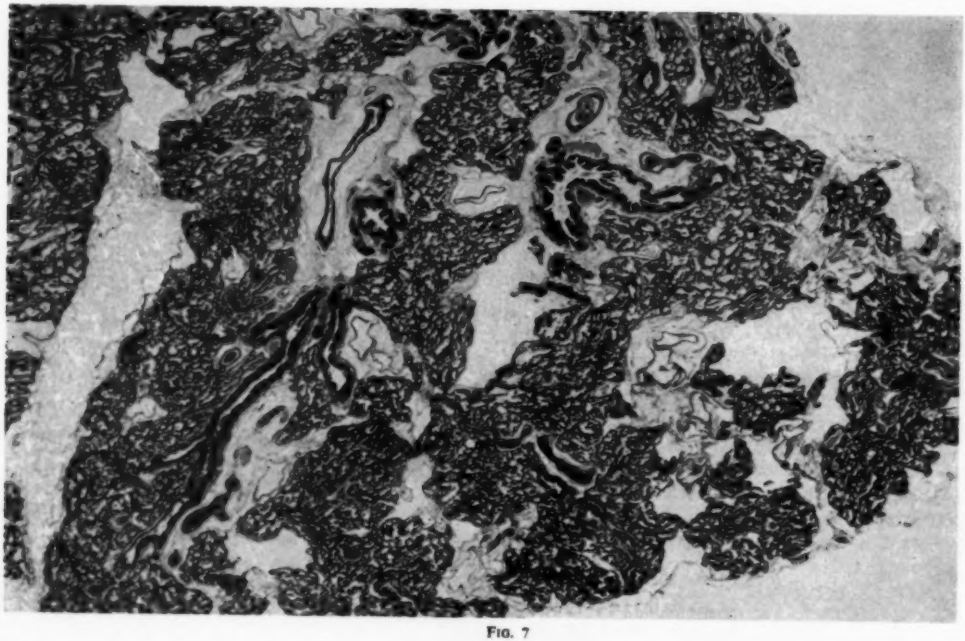

FIG. 7

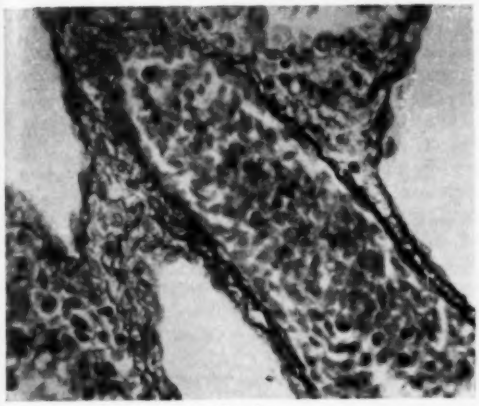

FIG. 9

FIG. 9.—A blood vessel running obliquely across the field with several lymphatic spaces in close relationship to it, and actually sharing a common wall (Weigert's elastic and Van Gieson's stain × 403).

FIG. 10.—Left lung (Case 4) showing subpleural and septal cystic spaces containing pus cells (haematoxylin and eosin × 70).

FIG. 11.—Reconstruction of 20 serial sections of 10 μ thick, with the outline of the cystic spaces drawn in indian ink, and the lumina lightly shaded in. The pleura and main blood vessels are lightly indicated. The lymphatic spaces are part of a continuous network of vessels.

FIG. 10

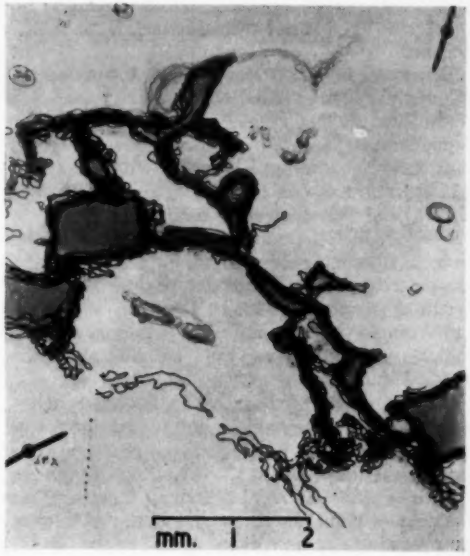

FIG. 11

and an occasional mononuclear cell. The bronchi generally have an intact epithelium. The hilar glands show no abnormality.

Discussion

The position of the cystic spaces in the normal distribution of lymphatics, as described by Miller (1947), their close relationship to blood vessels and bronchi, and their structures can leave little doubt that they must be derived from lymphatic vessels. These cystic spaces differ considerably from the cysts of bronchial origin, which are often air-filled, have a lining of bronchial or respiratory epithelium and are related to, if not in actual continuity with, the air passages. Occasionally, however, such air-filled cysts may be lined by flattened "endothelial" cells as in the cases of Flemming (1934) and Bowden (1948), but

careful search usually reveals groups of columnar or cubical cells, indicating the true nature of the cysts.

Congenital lymphangiectasis must be distinguished from a number of other conditions. At necropsy it could be mistaken for interstitial emphysema as happened in Case 3. In emphysema, however, the cystic spaces contain air, and under the microscope, although they may have an " endothelial " lining acquired from the connective tissue, neither their position and their relationship to other structures nor the structure of the wall is constant.

Care must be taken when airless lungs containing normal but dilated lymphatic vessels are examined, as they may occasionally have the honeycombed appearance of congenital lymphangiectasis. Closer examination will reveal that the vessels are fewer in number, constant in calibre, and run a straight course.

The rare intrapulmonary lymphangioma, though difficult to distinguish from lymphangiectasis, is recognized by its localized nature and its differing structure (Anderson, 1953). The case of Anspach and Wolman (1933) reported as pulmonary lymphangiectasis is almost certainly an example of one of these tumours.

Finally, the condition has to be differentiated from the cases in which pulmonary lymphatic cysts are part of a generalized lymphatic disorder throughout the body, such as those of Mann and France (1955) and McKendry, Lindsay, and Gerstein (1957). In congenital cystic pulmonary lymphangiectasis the cysts seem to be strictly confined to the lung.

As the condition appears to be found only in stillbirths and neonates, it can be assumed to be congenital. The only case reported in an older subject (Bredt, 1952, Case 2) is more likely to be one of bronchial cystic disease with the cysts having the endothelial-like lining referred to earlier. No blockage of the veins or lymphatic vessels has yet been seen to suggest secondary lymphangiectasis, while chylothorax, lymphorrhagia, and inflammatory reaction of the connective tissue amongst other features (Roujeau, Delarue, and Depierre, 1950) were not present in the cases examined.

On the contrary, it is much more likely to be a primary developmental anomaly similar to the lymphangiectasis in the limbs of newborn infants where neither hypertrophy of the vessel walls nor the tissue reaction is seen (Allen, Barker, and Hines, 1946). The occurrence of other major congenital malformations in over half the known cases is additional evidence in favour of this view.

A possible explanation of this anomaly has been suggested earlier (Laurence, 1955). The lymphatics, which grow into the lung bud about the ninth week of intrauterine life, have formed by the fourteenth week large trunks in the connective tissue septa, which divide the parenchyma into distinct lobules. Normally this lobulation becomes indistinct after about the twentieth week as connective tissue diminishes and the lymphatics become much narrower (Maximow and Bloom, 1942). It is suggested that pulmonary lymphangiectasis represents a continued growth of the lungs, with the tissue elements maintaining the proportions observed at 16 weeks. This seems to be the only satisfactory explanation for the unusual histological appearances.

Giammalvo (1955) maintains that cystic dilatation of the lymphatics results from failure or delay in the linkage of isolated lymphatic spaces. If this were so, cysts would be more likely to be confined to the peripheral portions of the lungs where fusion would occur last. This distribution was not evident nor were the spaces found to be isolated cysts in the numerous serial sections studied and reconstructed, but were shown to form a network of intercommunicating channels. Injection methods could establish this point, but so far there has been no opportunity, as the lungs have been fixed or extensively cut beforehand.

Progressive cyanosis accompanying the cystic condition is not difficult to explain. Of the cases described, three were accompanied by congenital heart disease, and two, or possibly three others, by intracranial haemorrhage. In the remaining three, where cyanosis must have been largely of pulmonary origin, there was little venous or bronchial obstruction from the grossly enlarged but undistended lymphatic channels, as in some cases of interstitial emphysema (Macklin and Macklin, 1944). It is also unlikely that the volume of the cysts produced poor aeration of the lung by taking up the available thoracic space. The bulky interlobular connective tissue and the cystic lymphatic vessels probably increased the resistance to lung expansion, while the inelasticity of the lungs decreased the pumping action from the respiratory excursions on the pulmonary circulation (Maidman and Barnett, 1957). This would explain the cardiac changes sometimes noted. In addition, the parenchyma in several instances was immature. Taken together, these factors could explain the progressive cyanosis and the rapid clinical deterioration.

Summary

A further seven cases of congenital cystic pulmonary lymphangiectasis are added to the three previously described in 1955 and the five others reported in the literature. Evidence is produced to show that the condition is a diffuse but irregular dilatation of the pulmonary lymphatic vessels. This form of pulmonary cystic disease is differentiated from other similar conditions.

It is suggested that this is a true developmental malformation and the possible pathogenesis is discussed. Probable reasons for the accompanying cyanosis are given. The evidence indicates that this malformation is more common than the few reported cases would suggest.

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Addendum

Since submitting the above paper, three further cases have appeared.

Case 11.—A 10 lb. male infant was cyanosed from birth. Resuscitation was without avail and he died after 20 hours. At necropsy the lungs showed gross interstitial emphysema, and the brain an intraventricular haemorrhage.

Case 12.—An 8 lb. 8 oz. male infant cyanosed from birth died at 27 hours. Necropsy revealed multiple malformations, especially of the heart. Bulky lungs and a tentorial tear were found.

Case 13.—A 6 lb. male infant cyanosed from birth died at nine hours. At necropsy the lungs showed naked eye lymphangiectasis.

In Case 11 the pulmonary lymphangiectasis was obscured by superimposed emphysema (possibly resulting from the efforts at resuscitation), but the underlying cystic condition was evident on microscopy. Case 12 also was a microscopic diagnosis, while in Case 13 the cysts were obvious at necropsy.

These additional cases were found by Dr. N. E. France, of the Queen Elizabeth Hospital for Children, Hackney, in a series of 249 perinatal necropsies. They support the contention that the condition is not uncommon.

J. clin. Path. (1959), **12**, 70.

THE INCIDENCE FOUND AT NECROPSY OF METASTASES TO THE STERNUM AND ILIAC CREST, WITH SPECIAL REFERENCE TO CARCINOMA OF THE LUNG

BY

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From Lewisham Group Laboratory, London

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Marrow samples from patients under investigation for anaemia only occasionally reveal metastatic malignant cells. Berkheiser (1955) detected malignant cells in 12 of 246 routine marrow aspirations from 241 patients, in most of whom the investigation was being done for the diagnosis of various types of anaemia. His series included six cases of multiple myeloma, a condition excluded from consideration in the investigation described in this paper. Berkheiser's other cases consisted of three of carcinoma of prostate, and one each of carcinoma of stomach, colon, and lung. Weisberger and Heinle (1949) sought for metastatic cells in the sternal marrow of patients known to have carcinoma and found them in seven, distributed as follows: two from 10 cases of carcinoma of breast, two from eight of carcinoma of lung, two from four of carcinoma of kidney, and one of five of carcinoma of prostate.

When malignant cells are found in routine diagnostic marrow smears it raises the question of the site of the primary growth.

It would be of value in approaching this problem to have some information as to the probability of particular malignant tumours metastasizing to those sites from which marrow biopsies are usually taken, namely, the sternum and iliac crest. While recognizing the shortcomings of necropsy statistics it was decided to search for metastatic malignant cells in the sternum in a series of necropsies on cases of malignant disease. Later in the investigation the iliac crest was sectioned also. It was hoped to obtain some data regarding the overall frequency of metastases to these sites in fatal cases of malignant disease, and during the investigation certain facts relating to carcinoma of the lung emerged which appeared to justify their particular consideration.

Material and Methods

The material was obtained from 225 unselected necropsies on cases of malignant neoplasm performed at Lewisham Hospital between 1954 and 1958. In three instances in this series double primary tumours were considered to be present. Malignant neoplastic conditions of the reticulo-endothelial system, myeloma, leukoses, and malignant tumours of the central nervous system were excluded.

For the first 98 cases the sternum only was sectioned; for the subsequent 127 cases both sternum and iliac crest were examined.

A thin slice from the midline of the manubrium sterni and a similar thin slice from the anterior 4 cm. of the anterior iliac crest were decalcified, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. By this histological method malignant cells are usually easy to identify by their tendency to occur in small clumps, usually demarcated from the surrounding marrow tissue, and sometimes with considerable associated stroma reaction. The degree of invasion varied from an occasional small group of cells to total replacement of marrow by neoplastic tissue.

Results

In 98 cases in which the sternum only was sectioned, malignant cells were found in 23. Of 127 cases in which both sternum and iliac crest were examined, malignant cells were found in 22: in both in 15 cases, in the sternum only in five cases, and in the iliac crest only in two cases (one case of carcinoma of stomach and one case of carcinoma of kidney). The overall picture is that, of 225 necropsies of cases of malignant disease, metastatic malignant cells were found in sternum or iliac crest, or both, on 45 occasions, an incidence of 20%. In all these instances metastases were found to be present in other sites as well; in five the skeleton was the only site of metastasis, in two cases of carcinoma of prostate, two of breast, and one of lung.

TABLE I
SITE OF PRIMARY TUMOUR IN 225 CASES

Site of Primary Tumour	No. of Cases	Sternal Metastases in 225 Examinations	Iliac Crest Metastases in 127 Supplementary Examinations
Lung	69	24	7
Stomach	37	4	2
Colon and caecum	21	2	1
Breast	17	5	2
Kidney	11	4	2
Prostate	9	2	2
Rectum	9	—	—
Pancreas	9	—	—
Ovary	6	—	—
Urinary bladder	6	—	—
Biliary tract	6	2	1
Liver (primary)	5	—	—
Uterus (carcinoma of body and of cervix)	5	—	—
Malignant melanoma of skin	3	1	—
Unknown	3	1	—
Sarcoma of uterus	2	—	—
Argentaffin carcinoma of ileum	2	—	—
Penis	1	—	—
Haemangiosarcoma of spleen	1	—	—
Melanoma of eye	1	—	—
,, oesophagus	1	—	—
,, larynx	1	—	—
,, thyroid	1	—	—
,, pituitary	1	—	—
Retroperitoneal sarcoma	1	—	—

The above figures include two cases of primary carcinoma of stomach with prostate, and one of argentaffin carcinoma of ileum with stomach, no marrow metastases being present in these cases.

Table I shows the site of the primary tumour in the 225 cases which form the source of material, and the number of positive findings in the sternum and iliac crest.

Carcinoma of Lung.—The 69 cases of carcinoma of lung examined during this investigation provide the largest single category of malignant neoplasm, and, with 24 instances of metastasis in the sternum, also provide the highest percentage of positive findings in this series, namely, 34.7%.

Certain cytological types of lung cancer appear more liable to metastasize in this way than others, as Table II indicates.

TABLE II
CYTOLOGICAL TYPES OF LUNG CANCER

Cell Type	No. of Cases	No. with Sternal Metastases
Oat-cell	26	14
Anaplastic pleomorphic	17	7
Squamous	16	Nil
Adenocarcinoma	10	3

An attempt was made to grade the degree of marrow invasion according to the arbitrary scheme:

+ =a single metastatic deposit
++ =a few separate metastatic deposits
+++ =extensive or complete replacement of marrow by metastatic tumour cells

It was apparent that the different cell types varied in the degree of metastatic replacement they achieved (Table III).

TABLE III
DEGREE OF METASTATIC REPLACEMENT ACCORDING TO CELL TYPE

Cell Type	Degree of Invasion of Marrow		
	+	++	+++
Oat-cell	3	3	8
Anaplastic	4	2	1
Adenocarcinoma	1	1	1

It appears that the oat-cell type of carcinoma is not only more liable to metastasize to the sternum (14 out of 26 cases—a remarkably high figure of 54%), but also is more likely to produce extensive replacement of the sternal marrow by tumour tissue. The practical importance of such extensive replacement is that it should make it more likely that malignant cells would be obtained in a diagnostic marrow sample from such patients.

Those cases of carcinoma of the lung showing sternal metastases were arranged in three groups according to the total duration of illness from onset to death as follows (in one case the clinical history was too vague to present this):

1 year to 6 months = 4 cases
5 months to 7 weeks =10 cases
6 weeks or less = 9 cases

Of the nine cases in which the duration was six weeks or less, in five the bone marrow was found to be almost completely replaced by malignant tissue (+++ grading on the above scheme).

This suggests that in suspected cases of carcinoma of the lung sternal puncture may be of value as a diagnostic procedure for the detection of malignant cells even when the clinical history is relatively short. Further, it might well be worth subjecting patients in whom thoracic surgery is contemplated to preliminary sternal puncture to try to determine whether or not skeletal metastasis has already taken place.

Miscellaneous Observations.—Apart from the cases of carcinoma of the lung, the examples of other forms of malignant disease in this series are too small to justify statistical appraisal, but it may be noted that the breast, kidney, and prostate feature as primary sites of origin for metastases to sternal and iliac marrow. This is similar to the experience of Weisberger and Heinle (1949). The discovery of metastases on five occasions in the small series of 37 cases of carcinoma of the stomach again draws attention to the tendency of this neoplasm to metastasize to bone, a fact, as

Meyer (1957) has pointed out, not very generally appreciated. It is of interest that one case of metastasizing malignant melanoma of the skin showed extensive infiltration of the sternal marrow at necropsy. This is regarded as rare. Meyer reported one case in his series of skeletal metastases.

Summary and Conclusions

The discovery of metastatic malignant cells in bone marrow samples frequently poses the question of their origin. An attempt has been made to assess the probability of particular tumours metastasizing to those sites from which bone marrow biopsies are commonly taken.

In a series of 225 cases of fatal malignant disease, excluding primary tumours of blood-forming organs, the sternum was examined at necropsy for the presence of malignant cells. In 127 cases of the series this was supplemented by examination of the iliac crest marrow. Metastases were found in one, or other, or both sites in 20% of the cases. In only two cases were metastases found in the iliac crest but not in the sternum.

Sixty-nine cases of carcinoma of the lung were included in the series and 34% showed sternal marrow metastases. With oat-cell carcinoma of lung, 54% were found to have metastasized to the sternum. Indirect evidence is adduced suggesting that extensive sternal metastases may be present in cases of carcinoma of the lung after a relatively short clinical illness. It is suggested that sternal marrow biopsy may have a place as a diagnostic and as a screening procedure in this condition.

The tendency of carcinoma of the stomach to metastasize to the skeleton is further confirmed.

I wish to express my thanks to Mr. H. Eastwood for his technical assistance in this investigation.

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THE PROBLEMS OF HISTOLOGICAL DIAGNOSIS IN BASO-SQUAMOUS CELL CARCINOMA OF THE SKIN

BY

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Following Jacob's clinical description in 1827 and Krompecher's histological investigations in 1900 and 1903, basal-cell carcinoma was regarded as a single clinical and pathological entity.

In 1910, however, MacCormac reported a series of rodent ulcers and suggested that on histological grounds there were three types of tumour, viz., pure rodent ulcers, mixed rodent ulcers (basal-cell and squamous-cell tumours side by side), and intermediate types in which "great difficulty was experienced in determining whether the neoplasm should be classified as a rodent ulcer or as a Malpighian cancer." Korbl (1912) subsequently described a transitional form of skin cancer having the structure of a typical basal-cell carcinoma, but in which the cells were larger, paler, and richer in cytoplasm, approaching the prickle cell type. In these tumours some of the cells were arranged concentrically and intercellular bridges were present.

Darier (1922), in discussing the problems of skin cancer, also drew attention to variations in the histological pattern of some basal-cell carcinomata and suggested that this group of atypical basal-cell tumours should be called "épithéliome métatypique," of which he recognized two types, "épithéliome métatypique mixte" and "épithéliome métatypique intermédiare." The former he described as having the configuration of a typical basal-cell carcinoma with areas of conglomerate squamous cells, pale, and acidophilic, often with prickles but without keratohyalin and surrounding a colloid centre. The intermediate type was described as having narrow trabeculae, two or three cells thick, the cells being larger and paler than basal cells, though not as large as prickle cells, and prickles were frequently absent.

Montgomery (1928), in a study of 119 cases of epithelioma of the skin, found 15 cases which he regarded as transitional between basal and squamous-cell carcinoma, and to these he applied the name "basal squamous cell epithelioma." He defined this term solely by citing case histories, though before detailing these he stated: ". . . but pearl formation, even though incomplete, or the appearance of prickle cells in places is not the picture belonging to a true basal cell epithelioma." In the case histories pearl formation—usually incomplete—with colloid-like material at the centre of these pearls, together with areas of large pale cells and occasional prickles, are the criteria on which the diagnosis of baso-squamous carcinoma was made.

Each of these authors stresses the importance of making the specific histological diagnosis of this type of tumour since radio-resistance and recurrence were thought to be common and metastasis likely, though none of them personally quotes cases in which metastasis had occurred.

These views seem to have been widely accepted at the time and little attention has been directed to this problem since the early descriptions. Lennox and Wells (1951) have recently made a study of differentiation in basal-cell carcinomata and concluded that it was doubtful if baso-squamous cell carcinoma was an entity. In the same journal, however, Thackray (1951) accepts baso-squamous cell carcinoma as an entity, though he does not define it.

This conflict of opinion is also expressed in the works of reference on which the average clinical pathologist has to rely when faced with a doubtful section. Thus Ackerman (1953) says that "practically all lesions diagnosed as baso-squamous carcinoma are basal-cell carcinoma and behave as such." Essentially similar are the views of Lever (1949, 1954), Evans (1956), and Allen (1953, 1957), who state that the appearances usually interpreted as baso-squamous cell carcinoma are simply areas of keratinization in basal-cell carcinomata and are of no prognostic significance. On the other hand, Barnard and Robb-Smith (1945) and MacLeod and Muende (1946) accept the existence of "metatypical carcinoma," and Willis (1953) refers to this

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type of tumour as "mixed basal and squamous-cell carcinoma." Each of these authors regards relative radio-resistance as a feature of this tumour.

It was because of this sharp difference of opinion between the authors quoted above and also because we were uncertain of the possible prognostic significance of the diagnosis of baso-squamous cell carcinoma that we undertook the present investigation. Interest was further stimulated by the knowledge that in the radiotherapy department here all cases diagnosed as baso-squamous cell carcinoma were treated as basal-cell carcinoma.

Material for this Study

Over the past seven years the diagnosis of baso-squamous cell carcinoma was made on 28 skin biopsies. In each case the original section stained with haematoxylin and eosin was available for study and the diagnosis was reviewed and amended where necessary. The criteria applied to each case and the results are shown in Table I. In selecting points for study in each section those features mentioned in the original papers on baso-squamous cell carcinoma, together with those in the paper by Lennox and Wells (1951) on differentiation in basal cell carcinomas, were noted. Attention was also paid to stromal fibrosis, since it was pointed out by Umiker and Director (1954) that fibrosing basal cell growths were often mis-diagnosed as baso-squamous cell carcinoma.

The follow-up on the cases has varied from six years to six months. Whilst it is appreciated that this period is too short so far as any worth-while assessment of recurrence is concerned, in each case we were in a position to judge the initial response to therapy, which is regarded as a significant point of difference between baso-squamous and pure basal-cell carcinomata.

Treatment was by a standard dose (4,000 r) of superficial *x* rays in 20 cases, the remaining eight cases being treated by biopsy excision.

Results and Discussion

At the outset of this study the immediate problem was that of defining baso-squamous cell carcinoma. A study of the literature unfortunately did not help. Indeed, it added to the confusion since, where illustrations have been given by authors, these seemed only to be examples of keratotic basal cell carcinomata (Montgomery, 1928; Willis, 1953). The only description which came near to being acceptable was that given by

TABLE I
CRITERIA FOR SELECTION AND RESULTS IN PRESENT SERIES

Case No.	Palisading	Whorls	Colloid	Keratohyalin	Keratinization	Prickles	Large Cells	Trabeculae	Fibrosis	Pigment	Secretion	Spindle Cells	Revised Diagnosis
1	−	+	−	−	−	−	+	−	−	−	+	+	Basal cell carcinoma
2	−	+	−	+	+	+	+	−	−	−	−	−	Seborrhoeic wart
3	+	−	−	−	−	−	+	+	+	−	+	+	Fibrosing basal cell carcinoma
4	−	+	−	+	+	+	+	−	−	−	−	−	Squamous cell carcinoma
5	−	+	−	−	+	+	+	−	−	−	−	−	Keratotic basal cell carcinoma
6	+	+	+	+	+	−	+	+	+	−	−	+	Basal cell carcinoma
7	+	+	−	+	+	−	+	−	−	−	−	−	Seborrhoeic wart
8	+	−	−	+	+	+	+	−	−	−	−	−	Mixed squamous and basal cell carcinoma
9	+	+	−	−	+	+	+	−	−	−	−	−	Pseudo glandular squamous cell carcinoma
10	−	+	−	+	+	+	+	−	−	−	−	+	Hyperkeratosis
11	−	−	−	−	−	+	+	−	−	−	−	−	Warty naevus
12	−	+	−	−	+	−	−	−	−	+	−	−	Keratotic basal cell carcinoma
13	+	+	−	−	+	+	+	−	−	+	−	−	Pseudo glandular squamous cell carcinoma
14	−	+	+	−	−	−	+	−	+	−	+	−	Keratotic basal cell carcinoma
15	+	+	+	−	+	−	−	−	−	−	+	−	,, ,, ,, ,,
16	−	−	+	−	−	−	−	+	+	+	+	−	Fibrosing ,, ,, ,,
17	−	−	+	−	−	−	+	+	+	−	+	+	Basal cell carcinoma
18	+	+	−	+	+	−	+	−	−	−	−	−	Keratotic basal cell carcinoma
19	+	−	−	−	−	−	−	−	−	−	+	−	Basal cell carcinoma
20*	+	−	−	−	−	+	+	+	+	−	−	−	Fibrosing basal cell carcinoma
21	+	+	−	+	+	+	+	−	−	−	−	−	Keratotic ,, ,, ,,
22	−	+	+	−	+	−	+	+	+	−	−	+	Fibrosing keratotic basal cell carcinoma
23	−	−	−	−	+	+	+	+	+	−	+	−	Baso-squamous cell epidermal tumour
24†	+	−	−	−	−	−	+	−	−	+	+	−	Basal cell carcinoma
25	+	−	−	−	−	−	+	+	+	−	+	+	Baso-squamous cell carcinoma
26	+	−	−	−	−	−	−	+	+	−	+	+	Fibrosing basal ,, ,,
27	−	+	−	+	+	−	−	+	+	−	+	+	Keratotic ,, ,, ,,
28	+	+	−	+	+	+	+	−	+	−	−	+	,, ,, ,, ,,

*Case 20: Prickles and large cells in hyperplastic epidermis. † Case 24: Large cells at junction with epidermis only.

MacCormac (1910), who stated: "This group includes a few growths where great difficulty is found in determining whether the neoplasm should be classified as a rodent ulcer or as a Malpighian cancer." Though this is an excellent assessment of the situation it remains too vague to be a definition.

For the purposes of this study baso-squamous cell carcinomata have been defined as that group of tumours which has the overall pattern of basal cell carcinoma and cytologically shows areas of basal cells differentiating into neoplastic squamous cells. In framing this definition we have been influenced by a number of considerations. Clearly, unless a tumour has some of the features of a basal cell carcinoma, consideration of a diagnosis of baso-squamous carcinoma is not likely to arise. Where squamous cell differentiation has occurred in a basal cell carcinoma, unless the squamous cells themselves show malignant features, they are unlikely to influence the prognosis of the tumour, and thus any diagnosis which draws attention to them would be therapeutically valueless. This view has been previously stated by Broders (1925), who pointed out that cells showing signs of keratinization are not capable of reproduction and that such cells are not active cancer cells. They cannot therefore of themselves be regarded as evidence of increased malignancy in any tumour in which they occur.

As defined here, a baso-squamous cell carcinoma is the malignant counterpart of the baso-squamous cell tumour (baso-squamous cell acanthoma) discussed and illustrated by Lund (1957). He describes this latter tumour as a benign epidermal tumour consisting of a haphazard mixture of small basal cells and larger squamous cells in irregular zones or rounded foci, inflammation of the connective tissue being variable though an almost constant feature. Khanolkar (1947) described such a tumour which he regarded as an epithelioma. In this series of cases there was one example of the baso-squamous cell tumour (Fig. 1).

Having formulated a definition, it is apparent that basal-cell carcinomata in which there are areas of keratinization, large pale acidophilic cells with or without prickles, and colloid degeneration of the centre of cell masses, are not to be classified as baso-squamous cell carcinomata, though it is realized that Montgomery (1928) would have done so. Likewise, Darier (1922) would have regarded these tumours as "épithéliome métatypique," and this view would be supported by Barnard and Robb-Smith (1945), MacLeod and Muende (1946), and Willis (1953).

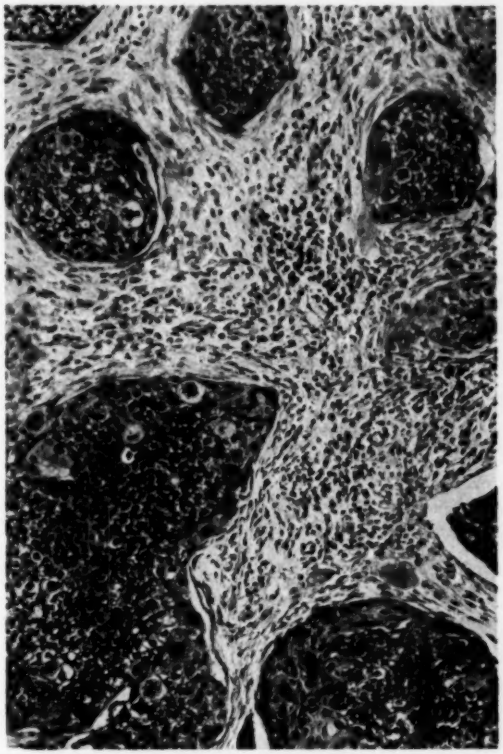

FIG. 1.—Baso-squamous cell tumour showing haphazard arrangement of basal and squamous cells with inflammation of stroma. (Haematoxylin and eosin, × 140.

Tumours in which there are separate foci of squamous and basal cell carcinoma should be described as such and should not give rise to difficulty in diagnosis or treatment (contiguous basal and squamous cell carcinoma).

The results of analysis of this series of tumours are given in Table II. The most

TABLE II

ANALYSIS OF TUMOURS

Keratotic basal cell carcinoma	9	Basal-cell carcinoma: 18
Fibrosing " " "	4	
Basal cell carcinoma	5	
Squamous cell carcinoma	3	
Contiguous basal and squamous cell carcinoma	1	
Baso-squamous cell carcinoma	1	
" " " tumour	1	
Seborrhoeic wart	2	
Warty naevus	1	
Hyperkeratosis of skin	1	

strange conclusion reached was that only one case out of the total of 28 could be regarded as a baso-squamous carcinoma as here defined. Even the case that has been accepted has much more the appearance of a mixed basal and squamous cell carcinoma (Fig. 2). However, as the two tumours are intimately mixed, in a few areas carcinomatous basal cells and squamous cells being continuous as distinct from contiguous, this case is acceptable as baso-squamous cell carcinoma (Fig. 3). A single case is not suitable for assessment of any therapeutic regimen. This case was treated by excision biopsy 15 months ago and there is no evidence of recurrence or metastasis to date.

With only one case of baso-squamous cell carcinoma to consider the natural history of this type of tumour cannot profitably be discussed. We would say, however, that in our experience it is a very rare tumour, constituting 0.018% of the 560 basal cell carcinomata seen in this department during the years covered by this study.

Though this study has not greatly advanced knowledge of baso-squamous cell carcinoma, a consideration of a number of the histological appearances present in this series of cases, with a possible explanation of the diagnostic errors which have been made, seems to be warranted. The four cases with benign epithelial hyperplasia are excluded from further consideration, though the fact that a diagnosis of baso-squamous cell carcinoma was applied to benign conditions signifies, amongst other things, some confusion as to what constitutes this tumour.

From Table II it can be seen that out of the original 28 cases 18 are reclassified as basal-cell carcinomas. In each of these cases a simple report of "basal-cell carcinoma of skin" or "rodent ulcer" should have been issued, and subdivisions have been made here purely for the purposes of discussion. That simple reporting, as indicated, is adequate is supported by the subsequent clinical course of the cases. Of the series, 10 cases were treated by radiotherapy and eight by excision. Only one case of undifferentiated basal-cell carcinoma showed delayed response to *x* ray, and even this lesion was completely healed within 10 weeks, and it had not recurred 10 months after treatment. Case 12 was treated by excision and recurred, but the section from the first excision indicates that it was incomplete and thus it is doubtful if this was a true recurrence. On each occasion the tumour was a basal cell carcinoma with areas of keratinization in it. The tumour in Case 27 recurred nine months after radiotherapy. The original tumour, which is included in this survey, was a basal cell carcinoma in which there were areas of keratinization; the recurrence was entirely undifferentiated. The number of cases in this series and the recurrence rate are too small to justify the drawing of firm conclusions. However, the recurrence of one case out of 10 after radiotherapy is comparable with a recurrence of 12.9% at three years in a series of cases treated by surface application of radium reported by Magnusson (1935). As already indicated, it is doubtful if the one recurrence after surgical excision represents true recurrence. Any figures relating to recurrence after excision are inevitably of doubtful significance since, whereas adequate excision is curative of the tumour in question, separate foci of development are often a feature of basal-cell growths.

Of the 18 cases now regarded as basal-cell carcinoma, nine showed areas of keratinization within the tumour cell masses (Fig. 4). This type of tumour is commonly referred to as keratotic basal-cell carcinoma, and it is this failure to appreciate the fact that keratotic foci are a feature of basal-cell carcinomas without prognostic importance which has led to the diagnostic errors in these cases and to the original descriptions of baso-squamous cell carcinoma. The significance of these keratotic foci remains in doubt. They do not appear to be cell nests comparable to those of squamous-cell carcinoma (Darier, 1922; Montgomery, 1928) or abortive hair follicles (Haythorn, 1931) or sweat duct orifices (Foot, 1947). For reasons already given it is impossible to envisage their having any adverse prognostic significance, and such has proved to be the case in this series. It is of interest to recall that this type of tumour is regarded as being radio-resistant, though radio-resistance was not encountered in this series. Dr. I. W. Whimster has kindly lent a section of such a tumour which has been irradiated with the resultant destruction of tumour cells, leaving minute horn cysts, i.e., micro-epidermoid cysts (Fig. 5). In such a situation there would be fine nodularity of the skin in the treated area, suggesting radio-resistance.

True prickles were detected in three out of the nine cases of keratotic basal cell carcinoma, and their presence no doubt confirmed the original observers' views that here was further evidence of squamous-cell carcinoma, though Dubreuilh and Auché reported the existence of prickles in basal cell tumours in 1901. Pseudo prickles, which appear to be the result of secretory activity of the

FIG. 2.—Baso-squamous cell carcinoma. At the edge of the lesion the appearance is that of a trabecular basal cell carcinoma. This tissue blends with spindle cell squamous carcinoma in which individual cell keratinization is occurring. (Haematoxylin and eosin, × 40.)

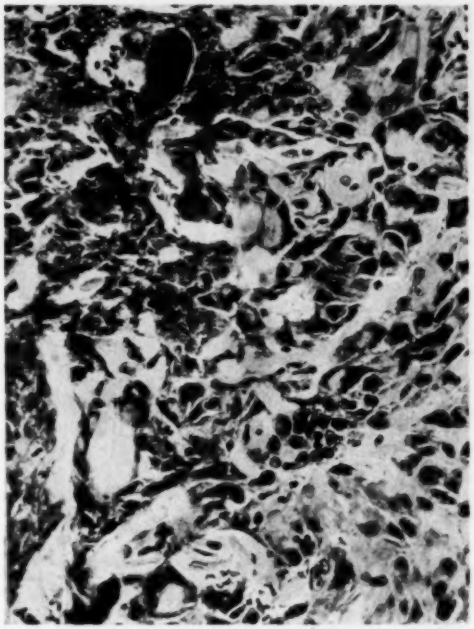

FIG. 3.—Baso-squamous cell carcinoma. High-power view of Fig. 2. (Haematoxylin and eosin, × 270.)

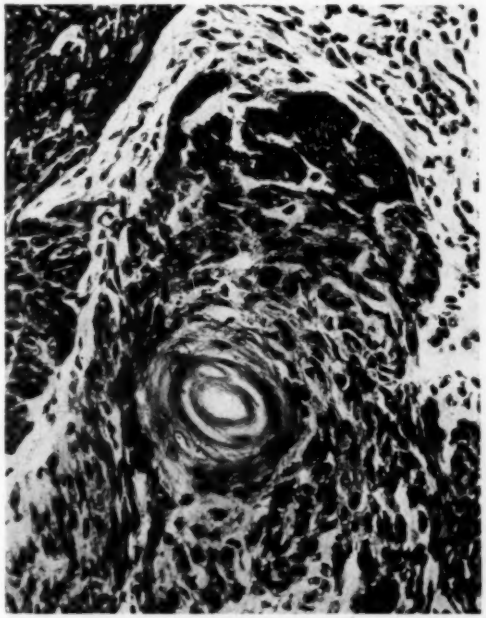

FIG. 4.—Keratotic basal cell carcinoma. An area of keratinization is present to one side of the centre of a clump of basal cells. This change was not present in adjacent areas. (Haematoxylin and eosin, × 270.)

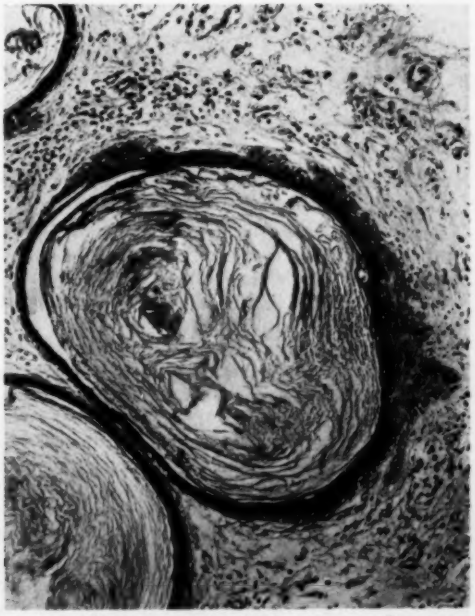

FIG. 5.—Irradiated keratotic basal cell carcinoma. The tumour tissue has been destroyed leaving a small hair cyst surrounded by normal squamous cells. (Courtesy of Dr. I. W. Whimster.) (Haematoxylin and eosin, × 120.)

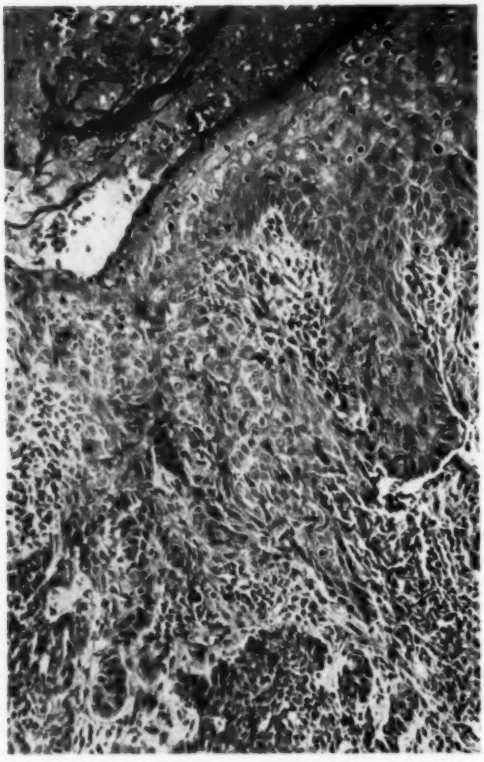

FIG. 6.—Pseudo epitheliomatous hyperplasia at the edge of an ulcerated basal cell carcinoma. (Haematoxylin and eosin, × 140.)

tumour cells (Lennox and Wells, 1951), are often seen and they constitute a trap for the unwary.

Four cases in the basal cell group showed a marked connective tissue response to the invasion of the tumour. The resultant microscopic picture is of a trabecular infiltrative type of tumour in which it is difficult to delineate the typical basal cells from the larger eosinophilic fibrocytes, these latter being liable to interpretation as squamous cells. Attention was drawn to this diagnostic difficulty by Umiker and Director (1954), who showed that this appearance had no prognostic significance. Once aware of the appearances of these fibrosing basal cell growths, no difficulty is encountered in interpreting them.

One final appearance, which was the cause of some difficulty and led to erroneous diagnoses, was hyperplasia of squamous epithelium at the edge of ulcerated lesions. Such epithelial hyperplasia is of course not uncommon at the edge of chronic skin ulcers, and it is mentioned specifically at the edge of rodent ulcers by Thackray (1951) and by Lund (1957), the former author regarding it as squamous metaplasia of tumour cells. On general grounds, however, it is more likely to represent reactive hyperplasia of the squamous epithelium at the edge of the ulcer. Cases 17, 20, and 24 showed this appearance, and in each this was the only evidence of any squamous element in the tumour (Fig. 6).

Brief reference should be made to the three cases of squamous-cell carcinoma which gave rise to diagnostic difficulty and error. Two of the three cases were of pseudo glandular type—clumps of epithelium with low cuboidal cells with hyperchromatic nuclei at the periphery and larger eosinophilic cells at the centre, many of these latter cells appearing to have been desquamated—which perhaps bears a remote resemblance to a keratotic basal-cell carcinoma. The third case of squamous-cell carcinoma was an intra-epidermal tumour showing early invasion, and it is not easy to see what the diagnostic difficulty was in relation to the problem.

Summary and Conclusions

A histological diagnosis of baso-squamous cell carcinoma of the skin had been made on 28 biopsies in this department in the seven years 1951–7. Each section was reviewed.

The unsatisfactory descriptions and definitions in the literature are discussed and a definition proposed.

Using this definition, only one case could be regarded as a baso-squamous cell carcinoma. As the total number of basal-cell carcinomata seen in this department during the years covered by the survey was 560, this makes an incidence of only 0.018%.

The possible causes of erroneous diagnosis are discussed with a consideration of certain appearances in basal and squamous cell carcinomata.

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THE COLORIMETRIC ESTIMATION OF ALPHA-AMINO NITROGEN IN TISSUE FLUIDS

BY

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Various authors have reported methods for the colorimetric estimation of alpha-amino nitrogen with ninhydrin (Moore and Stein, 1948, 1954; Smith and Agiza, 1951; Troll and Cannan, 1953; Yemm and Cocking, 1955; Kalant, 1956; Rosen, 1957). Only Kalant has applied this method to the direct measurement of alpha-amino nitrogen in biological fluids; he reported on the determination of total alpha-amino nitrogen in dog plasma.

This paper describes a colorimetric ninhydrin method for amino-acid determination, based on that of Troll and Cannan. It differs in using disodium ethylenediamine tetra-acetic acid (E.D.T.A.) instead of pyridine to enhance colour development. This improves reproducibility by eliminating trace metal interferences, and yields lower blanks than the pyridine reagent. In addition, we use a separate buffer to maintain the *p*H optimal at 5.1. For the first time a technique has been evolved for applying the colorimetric ninhydrin procedure to biological fluids generally, and, in particular, it has been successfully applied to urine.

The optimum conditions for *p*H and concentration of cyanide, ninhydrin, and other reagents have been studied, and the optimal time for heating has been established. With the exception of the E.D.T.A. concentration, all the factors are moderately critical.

A: Method for Simple Solutions of Amino-acids

Buffer.—Add 300 ml. of 2M-sodium acetate (164 g./l. anhydrous salt) to 700 ml. of 2M-acetic acid (120 ml. glacial acetic acid/l.). Add approximately 5 g. of disodium ethylenediamine tetra-acetate and exactly 240 mg. of potassium cyanide. Preserve with chloroform. This solution is stable indefinitely.

Phenol Reagent.—Dissolve 400 g. of phenol in 100 ml. of absolute ethanol by heating. Cool and add approximately 5 g. of "permutit" (Decalso F). Stir well for 10 minutes and filter. Even moderate colour due to oxidation of the phenol does not interfere with subsequent determinations.

Ninhydrin.—Dissolve 6 g. of ninhydrin in 100 ml. of absolute ethanol by vigorous stirring. Add approximately 2 g. of "permutit" and stir for 10 minutes. Filter and store in a brown bottle. The solution is a pale yellow-green. If the solution is contaminated by protein or amino-acid the colour will change.

Stock Standard.—Any full-colour amino-acid is suitable (Table I). Monosodium glutamate (mol. wt. 187.14) has been used in this laboratory. Dissolve 935.7 mg. in distilled water and make up to 100 ml. This solution is 0.05 *M*. Preserve with chloroform.

TABLE I

COLOUR VALUES OF AMINO-ACIDS AND RELATED COMPOUNDS RELATIVE TO GLUTAMIC ACID ON A MOLAR BASIS

Full (97–103%)	High	Low
Glycine	Lysine 110%	Phenylalanine 94%
Alanine	Ornithine 110%	Tyrosine 88%
Leucine	Cystine 121%	Tryptophan 78%
Isoleucine		Taurine 29%
Valine		Beta alanine 8%
Serine		Ammonia nitrogen 46%
Histidine		Ethanolamine 16%
Citrulline		Ethylenediamine 16%
Threonine		Methylamine 8%
Artinine		Ethylamine 3%
Methionine		
Aspartic acid		Urea 0·09%
Asparagine		Creatinine 0·26%
Glutamine		Uric acid 0·00
Cysteic acid		Hippuric acid 0·07% or less

Working Standard.—Dilute stock standard 1 in 100 with distilled water. One millilitre of the working standard contains 7.0 µg. alpha-amino nitrogen.

Procedure.—Add 0.5 ml. of the unknown solution (containing not more than 3.5 µg. of alpha-amino nitrogen) to a 6×¾ in. test-tube. For the blank use 0.5 ml. ammonia-free distilled water, and for the standard take 0.5 ml. of the working standard (3.5 µg. of alpha-amino nitrogen). Add 0.5 ml. of buffer solution to each tube. Add 5 ml. of phenol reagent and shake until clear. Add 0.5 ml. of ninhydrin solution and shake again. Place the tubes in an actively boiling water bath for four to five minutes. It is unnecessary to stopper the tubes. Remove and cool in

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water for at least 10 minutes. Shake the tubes vigorously and equally. After shaking, the blank solution takes on a pale yellow colour, and the test solutions take on the typical purple colour. The colour fades slowly and proportionately for several hours. Allow the tubes to stand for at least 10 minutes after shaking, and read the optical density at 570 mμ or with a yellow filter (Ilford 626). When read against water, the optical density of the blank is always below 0.05.

The standard deviation of 100 consecutive duplicate determinations was ±2.10%. In Table I are listed the relative colour values of the common amino-acids and several other compounds. Proline, hydroxyproline, and cysteine have not been included because they do not give purple colours with ninhydrin (Moore and Stein, 1948).

B : Method for Plasma

Kalant (1956) found that ethanol, trichloro-acetic acid, and sulphosalicylic acid were unsatisfactory as protein-precipitating agents, but that tungstic acid was suitable. Acetone has been tested as a precipitant, but it is unsatisfactory because of the low solubility in acetone of many amino-acids. Picric acid is a satisfactory precipitant, but a considerable amount of extra labour is involved, since the excess picric acid must be removed (Stein and Moore, 1954). Tungstic acid was selected for this study. It is not ideal because it leaves a slight residual turbidity, which necessitates that the blank and standard also be treated with the protein precipitant.

Heparinized plasma was used. Serum is, of course, unsatisfactory owing to the considerable proteolysis which occurs on clotting. Separated plasma is stable indefinitely if frozen.

Protein Precipitant.—Add 1 part of 13.3% (w/v) sodium tungstate to 9 parts of 0.11 N-sulphuric acid (3.1 ml. of conc. sulphuric acid/l.). This mixture should be made up every few days as it rapidly becomes turbid.

Working Standard.—Dilute 4 ml. of stock standard to 100 ml. with distilled water.

Procedure.—Rinse 0.3 ml. of plasma into 0.7 ml. distilled water in a centrifuge tube. Add 3 ml. of the tungstic acid reagent with shaking. Centrifuge. Decant the supernatant through a cotton-wool plug. For the blank, add 3 ml. of the tungstic acid reagent to 1 ml. of ammonia-free distilled water, and for the standard add 3 ml. of tungstic acid reagent to 1 ml. of the working standard. In each case, analyse 0.5 ml. as in section A, reading the optical density against water. The optical density of the blank is always below 0.075.

Calculation.—The sample analysed is equal to 300/8 μl. of plasma; the standard analysed contains 3.5 μg. alpha-amino nitrogen; and μg. per 100 μl. equals mg. per 100 ml.

$$\frac{\text{O.D.* test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times \frac{8}{3} \times 3{\cdot}5 = \text{mg. alpha-amino nitrogen per 100 ml. plasma}$$

* O.D. = optical density.

Results.—The plasma of 29 normal laboratory workers was investigated by this method. Blood was drawn three hours after a light breakfast.

Fifteen Women.—Range 3.68–5.47 mg./100 ml., mean 4.52 mg./100 ml., S.D. ±0.51 mg./100 ml.

Fourteen Men.—Range 3.68–5.25 mg./100 ml., mean 4.56 mg./100 ml., S.D. ±0.40 mg./100 ml.

Combined Series.—Range 3.68–5.47 mg./100 ml., mean 4.54 mg./100 ml., S.D. ±0.45 mg./100 ml.

All specimens were analysed in duplicate and the standard deviation of the difference was ±3.7%.

From a patient with pernicious anaemia, 24 plasma samples were analysed over a period of six weeks. All of the values obtained fell between 3.86 and 5.23 mg./100 ml.

Recoveries of pure amino-acids added to plasma specimens on four occasions were 97%, 102%, 96%, and 103%.

C : Method for Cerebrospinal Fluid

The reagents are those for plasma. Rinse 0.6 ml. of cerebrospinal fluid into 0.4 ml. of distilled water in a centrifuge tube. Continue exactly as for plasma, preparing the blank and standard in the same manner.

Calculation.—The sample analysed contains 600/8 μl. cerebrospinal fluid. Hence,

$$\frac{\text{O.D.* test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times \frac{8}{6} \times 3{\cdot}5 = \text{mg. alpha-amino nitrogen per 100 ml. C.S.F.}$$

* O.D. = optical density.

Results.—Five fresh specimens of cerebrospinal fluid normal with respect to protein and cell content were investigated. The values were 1.08, 1.39, 1.44, 1.45, and 1.25 mg./100 ml.

D : Method for Urine

The principal problem in measuring urinary amino-acids by this method is completely to rid the sample of ammonia. This can be achieved by adding alkali to the urine sample, and evaporating to dryness over concentrated sulphuric acid.

For free alpha-amino nitrogen, the reagents are as follows:

Alkali Solution.—0.05 M-potassium carbonate (6.9 g./l. of the anhydrous salt).

Working Standard.—Dilute stock standard 1 : 1 with distilled water.

Procedure.—Rinse 0.2 ml. urine into 2 ml. 0.05 M-potassium carbonate solution in a small beaker. For the blank, substitute 0.2 ml. distilled water for the urine, and for the standard, substitute 0.2 ml. of the working standard. Place all the beakers in a large vacuum desiccator, the bottom of which contains 300–400 ml. of concentrated sulphuric acid. Evacuate the desiccator to a pressure of 20–30 mm. Hg. Allow the solutions in the beakers to go to dryness. This takes several hours, but it is most convenient to do this step overnight. Dissolve the residue in 10 ml. distilled water. Take 0.5 ml. of each for analysis as in section A. The optical density of the blank is always below 0.05.

Calculation.—The sample analysed contains 10 μl. of urine, and the standard 3.5 μg. of alpha-amino nitrogen. Thus,

$$\frac{\text{O.D.* test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times 10 \times 3{\cdot}5 = \text{mg. free alpha-amino nitrogen per 100 ml. urine}$$

* O.D. = optical density.

Results and Discussion.—This method was applied to 24-hour urine collections from 20 normal laboratory workers. The specimens were preserved with 6 ml. each of toluene and chloroform, and stored at 4° until analysed. Results are given in Table II.

TABLE II

URINARY FREE, TOTAL, AND BOUND ALPHA-AMINO NITROGEN IN 20 NORMAL PEOPLE

Urinary Alpha-amino Nitrogen		Range	Mean (±S.D.)	Significance of Difference between Male and Female Values
Free				
Concentration (mg./100 ml.)	8*	8·06– 15·54	10·73	
	12†	6·95– 17·52	10·14	
	combined	—	10·37	
Total (mg./24 hours)	8	75·8 –197·1	130·9 ±39·9	
	12	78·0 –203·1	132·2 ±35·1	
	combined	—	131·7 ±37·1	
Mg./kg./24 hours	8	1·39– 3·44	2·22± 0·63	
	12	0·98– 3·27	1·88± 0·63	P = 0·26
	combined	—	2·01± 0·67	
Total				
Concentration (mg./100 ml.)	8	14·51– 29·34	22·66	
	12	14·24– 31·60	23·33	
	combined	—	23·06	
Total (mg./24 hours)	8	192·0 –426·2	274·2 ±70·2	
	12	186·4 –423·0	299·4 ±66·2	
	combined	—	289·3 ±69·0	
Mg./kg./24 hours	8	3·52– 7·44	4·67± 1·16	
	12	2·75– 6·20	4·15± 0·97	P = 0·32
	combined	—	4·36± 1·07	
Bound				
Total (mg./24 hours)	8	81·8 –229·1	143·3 ±46·2	
	12	100·9 –259·6	167·2 ±44·2	
	combined	—	157·6 ±45·0	
Mg./kg./24 hours	8	1·24– 4·00	2·45± 0·79	
	12	1·62– 3·06	2·28± 0·44	P = 0·56
	combined	—	2·35± 0·60	
Ratio				
Total/free (mg./24 hours)	8	1·50– 2·67	2·17± 0·39	
	12	1·65– 2·82	2·32± 0·36	P = 0·41
	combined	—	2·26± 0·38	

* Female. † Male.

The alkali solution employed decreases the anticipated colour of the standard by about 5%, an effect not present when a weaker alkali solution is substituted. Using the 0.05 M-carbonate solution, up to 15 times the amount of ammonia normally present in urine may be completely removed. Since the maximum urinary ammonia may exceed the average by 10 to 12-fold, it seems advisable to use the 0.05 M-solution. Studies at various alkali concentrations have shown that standard and test solutions are proportionately affected, and the analytical results are unchanged. Recovery of pure amino-acids added to urine on five successive occasions gave results of 104%, 101%, 95%, 99%, and 102%.

The 20 specimens listed were analysed in duplicate, with a standard deviation of the difference of ±2.8%.

From the same patient mentioned earlier, seven 24-hour collections were made over a two-week period. The free alpha-amino nitrogen ranged from 74.6 to 99.7 mg. per 24 hours.

Total Alpha-amino Nitrogen

After acid hydrolysis, the urine ammonia level may rise over 50-fold due to decomposition of urea. Consequently, a higher alkali concentration is necessary for complete removal of ammonia by diffusion-absorption.

Alkali Solutions.—These are 0.40 M-potassium carbonate (55.2 g./l. of the anhydrous salt), and 0.25 M-potassium carbonate (34.6 g./l. of the anhydrous salt).

Working Standard.—This is the same as for free alpha-amino nitrogen.

Procedure.—To 2 ml. of urine in a $6 \times \frac{1}{2}$ in. "pyrex" test-tube add 1 ml. of concentrated hydrochloric acid. Seal the tube in a gas-oxygen flame and hydrolyse in an oven at 150° overnight. Allow to cool. Open the tube cautiously and remove the humin by decanting the hydrolysate through a cotton-wool plug.

Rinse 0.15 ml. of the hydrolysate into 2 ml. of 0.40 M-carbonate solution in a small beaker. At this point prepare the blank and standard by adding 0.2 ml. distilled water and 0.2 ml. working standard respectively to 2 ml. of 0.25 M-carbonate solution in individual small beakers. Proceed now exactly as listed under free alpha-amino nitrogen.

Calculation.—As 150 μl. of hydrolysate equals 100 μl. of the original sample, and 5 μl. of urine is in the final sample, therefore,

$$\frac{\text{O.D.* test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times 20 \times 3{\cdot}5 = \text{mg. total alpha-amino nitrogen per 100 ml. of urine}$$

* O.D. = optical density.

Results and Discussion.—The 20 normal urine specimens were also analysed for total alpha-amino nitrogen: the results are given in Table II. The standard deviation of the variation of the duplicate estimations was ±3.2%. The seven specimens from the patient mentioned earlier showed values between 266.0 and 303.2 mg./24 hours.

The higher alkali concentration used for the test solution is calculated to equal the concentration employed for the blank and standard solutions after neutralization of the hydrochloric acid.

As noted for free alpha-amino nitrogen, treatment with alkali produces fading of the standard, generally from 8 to 10%. For the reasons given earlier, the analytical results are unaffected, and two recoveries with pure amino-acids added to urine before hydrolysis were 98% and 99%.

Hydrolysis at 150° gave results about 5% higher than hydrolysis at 125°.

When glutamic acid and glycine were hydrolysed under the conditions above, there was a 4 to 5% loss. It was thought improbable that the various

amino-acids in the hydrolysed test solution would behave quantitatively similarly, and there would still exist uncertainty about the actual extent of amino-acid destruction. For this reason the standard was not routinely brought through the hydrolysis procedure. Consequently it is quite possible that the values for total alpha-amino nitrogen are underestimated to the extent of a small percentage.

Discussion

The standard procedure for determining plasma alpha-amino nitrogen is the ninhydrin-CO_2 method of Hamilton and Van Slyke (1943). Normal values by this technique have been published by the originators and by a number of other investigators (Woodruff and Man, 1945; Bonsnes, 1947; Brewer, Brown, Harvey, and Horwitt, 1947; Burt, 1953). The values given for plasma range from 3.3 to 5 mg./100 ml., with a mean of 4.1 to 4.2. The present method gives a mean and range about 10% higher than the gasometric technique. While the manometric method is probably more specific, the colorimetric procedure is far simpler and seems adequate for clinical applications.

The few figures presented here on alpha-amino nitrogen in cerebrospinal fluid agree with those of Christensen, Cooper, Johnson, and Lynch (1947), who report ninhydrin-CO_2 determinations on six specimens, with results ranging from 1.04 to 1.43 mg./100 ml.

Concerning urine levels, accurate data are limited. Thompson and Abdulnabi (1950), using the ninhydrin-CO_2 technique, give the average 24-hour output of free alpha-amino nitrogen as 126 ± 67 mg. for females (11) and 101 ± 39 mg. for males (13), with a combined mean of 113 mg./24 hours.

Eckhardt and Davidson (1949), by the same method, studied one case on varying protein intake, and found levels ranging from 63 mg. to 140 mg./24 hours. On a moderate protein intake the excretion was 118 mg./24 hours.

The gasometric method used in the two studies cited above does not estimate taurine (Van Slyke, Dillon, MacFadyen, and Hamilton, 1941). If one wishes to include this compound, which is excreted in large amounts, about 17 mg./24 hours must be added to the previous figures (Stein, 1953).

Stein (1953), using column separation and colorimetric ninhydrin estimation, has given figures for free urinary amino-acids on six normals. The values range from 97 to 145 mg. alpha-amino nitrogen/24 hours, with a mean of 120.

The present series gives a combined mean of 131.7 ± 31.1 mg. free alpha-amino nitrogen/24 hours, with a range of 75.8 to 203.2. It must be recalled (Table I) that only about one-third of the taurine is estimated by the present method. This error is roughly cancelled out by the error due to urea (see below). In view of the difference in technique and material, and the relatively small series reported in every case, the agreement between the previous and the present data is good.

Concerning the total alpha-amino nitrogen of urine, Thompson and Abdulnabi (1950) report the 24-hour output as 321 ± 138 mg. for females and 251 ± 66 mg. for males, with a combined mean of 283 mg./24 hours. Eckhardt and Davidson (1949) report a value of 575 mg./24 hours on their single case while on a moderate protein intake. Stein (1953) gives results on two cases as 356 and 455 mg./24 hours respectively.

The present series gives a mean of 289.3 ± 69 mg./24 hours, with a range of 186.4 to 426.2. In the present series, unlike the results reported by Thompson and Abdulnabi (1950), no significant sex difference was found, either for free or total alpha-amino nitrogen, when the results were calculated on a weight basis.

The data in Table I show that urea, creatinine, and hippuric acid all react to a slight extent. It can readily be calculated that the interference of the last two, even when present in abnormally large amounts, is negligible. With average urinary outputs of amino-acid and urea, the positive error due to urea is about 5%. If the amino-acid excretion were very low and the urea excretion very high, the error could reach 10%. In the reverse case of a low urea and high amino-acid excretion, the error would fall to about 2%. The error due to urea does not affect the results for total alpha-amino nitrogen, for urea is destroyed by the hydrolysis.

No attempt was made to remove urea from the sample before testing, or to correct for the actual urea content, because the principal use of a method for measuring free urinary amino-acids is in the screening for, and investigation of, cases of pathological amino-acid urea. In cases where the free amino-acid excretion is above the normal range, the error due to urea will drop to 1% or less, and may be ignored.

Summary

A simple variation of the colorimetric ninhydrin technique for estimating alpha-amino nitrogen is presented. Its application to plasma, cerebrospinal fluid, and urine is described. A series of normal values for these fluids obtained by the

present method is given. The present technique gives approximately the same values as other methods already described.

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A METHOD FOR THE DETERMINATION OF TARTRATE-LABILE, PROSTATIC ACID PHOSPHATASE IN SERUM

BY

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L(+)-Tartrate strongly inhibits prostatic acid phosphatase (Abul-Fadl and King, 1948, 1949) while having no action on red cell phosphatase. The authors suggested the application of this phenomenon and its diagnostic value as a sensitive test in elucidating doubtful or borderline cases of prostatic cancer. Based on this principle, Fishman and Lerner (1953) described a method for the determination of serum acid phosphatase of prostatic origin. This method requires larger amounts of serum, besides involving the precipitation and separation of proteins, and is more complicated and time-consuming than the method in use here.

The method now described is the result of further work on the tartrate-labile phosphatase component and is a modification of the amino-antipyrine (A.A.P.) methods in use in this laboratory (Kind and King, 1954). For borderline cases it probably gives a truer measure of the prostatic part of the serum acid phosphatase than does the formol-stable fraction. A somewhat similar procedure has been described by Davis and Ward (1956).

Reagents

Citrate Buffer.—Crystalline citric acid, 21 g., is dissolved in water, 188 ml. of N-NaOH added, the *p*H adjusted to 4.9 by cautious addition of N-NaOH or N-HCl, and made up to 500 ml. Preserve with a few drops of chloroform in the refrigerator.

Substrate (0.01 M-Disodium Phenyl Phosphate).—For the substrate, dissolve 2.18 g. 0.01 M-disodium phenyl phosphate in 1 litre of freshly boiled and cooled distilled water and preserve with a few drops of chloroform in the refrigerator.

Stock Phenol Standard (1 mg./ml.).—Pure crystalline phenol, 1 g., is dissolved in a litre of 0.1 N-HCl and preserved in the refrigerator.

Dilute Phenol Standard (0.01 mg./ml.).—One millilitre of stock standard diluted to 100 ml. with distilled water is preserved with a few drops of chloroform in the refrigerator.

0.5 N-Sodium Hydroxide.—This is stored in a polythene bottle and filtered when necessary.

0.5 M-Sodium Bicarbonate.—Sodium bicarbonate, 4.2 g., is dissolved in water and made up to 100 ml. The solution is stored in a polythene bottle and filtered when necessary.

4-Amino-antipyrine (A.A.P., 0.6%).—4-Amino-antipyrine, 0.6 g., is dissolved in water and made up to 100 ml. This solution should be stored in a brown bottle in the refrigerator and filtered before use.

A.A.P. Reagent.—A mixture of 0.5 M-$NaHCO_3$ and 0.6% 4-amino-antipyrine is made in the proportion of 1:1. The mixture is stored in the refrigerator in a brown bottle and is stable for a month.

Potassium Ferricyanide.—This reagent is made up as 2.4 g. per 100 ml. in water and kept in a brown bottle.

M-Tartrate.—L(+)-Tartaric acid (AnalaR), 15 g., is dissolved in about 70 ml. of water, 18.5 ml. of 10 N-NaOH added, the *p*H adjusted to 4.9 and made up to 100 ml. Stored in the cold room (4° C.) or refrigerator with a few drops of chloroform, this solution is stable. A few crystals of Na-H-tartrate may form if the temperature goes below 4° C.: these will re-dissolve on slight warming.

It is essential that all solutions used are optically clear.

Experimental

Concentration of Tartrate. — Experiments were carried out with diluted prostate extracts and seminal fluid to determine the optimum molarity of tartrate necessary completely to inhibit the prostatic component of acid phosphatase in the ranges usually encountered in blood plasma, including the higher levels found in metastatic carcinoma of the prostate. The dilutions were made with citrate buffer, as the diluted enzyme is more stable at the acid *p*H. The acid phosphatase activities of suitably diluted prostate extracts and seminal fluid were determined along with the activities in buffer-substrate mixtures containing tartrate of varying concentrations to give final molarities for tartrate ranging from 0.1 M to 0.0001 M. The buffer substrate mixtures were prepared by serial dilution of the stock molar tartrate with citrate buffer.

The results are shown graphically (Fig. 1). It is clear that for acid phosphatase activities in the ranges

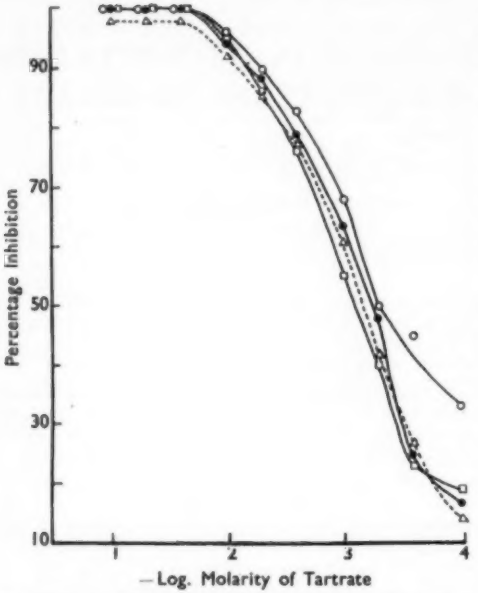

FIG. 1.—Tartrate inhibition of prostatic acid phosphatase. O—O—O diluted prostate extract (18 K.-A. units); ●—●—● diluted prostate extract (49 K.-A. units); □—□—□ diluted seminal fluid (131 K.-A. units); Δ—Δ—Δ serum from a patient with metastatic carcinoma of prostate (30 K.-A. units total, 29·2 units prostatic).

usually encountered in prostatic cases a final concentration of tartrate of about 0.025 M in the substrate-buffer-enzyme mixture is the optimum to give complete inhibition of the prostatic acid phosphatase, under the conditions of the test. Increasing the concentration of the tartrate beyond this serves no purpose (see also under Discussion). This was confirmed by a similar experiment on a serum of high prostatic acid phosphatase activity from a case of carcinoma of the prostate with metastases. The inhibition curve for serum follows the same pattern as the curves obtained for diluted prostate extract or seminal fluid. There is no further inhibition for molarities of tartrate greater than 0.025 M.

Recovery experiments in which diluted prostate extract or seminal fluid was added to several specimens of serum also confirmed the above finding, i.e., that a final concentration of about 0.025 M-tartrate was the optimum for complete inhibition.

Stability of Acid Phosphatase.—Under many conditions prostatic acid phosphatase is a very unstable enzyme. An aqueous prostatic extract loses its activity very rapidly if left out at room temperature. The same thing is true of the total, as of the prostatic, acid phosphatase activity of serum. A serum from a case of prostatic carcinoma with metastases, which had total and prostatic acid phosphatase activities of 62 and 60 units respectively while fresh, gave total and prostatic activities of only 1 and 0 respectively after standing at room temperature (ca. 20° C.) for four days. This almost complete destruction of enzyme can occur sometimes in as little as 24 hours, when serum is left at room temperature. A specimen which had an activity of 76 units showed an activity of only 4 units when left at room temperature for just over 24 hours. In another case a serum which had an extremely high activity of 956 units came down to 180 units in 48 hours. Daniel (1954) found that the *p*H of serum changes from 7.4 immediately after separation to 7.5 in 2 min., 7.8 in 5 min., 8.0 in 15 min., 8.4 or more in 24 hours, when left at 24° C. Serum in contact with its clot remained at *p*H 7.4 for a considerable time, and under these conditions the acid phosphatase was stable at temperatures below 30° C. for periods up to 24 hours.

The formol-stable as well as the tartrate-labile acid phosphatase activity of serum left in the refrigerator immediately after separation appears to be practically the same as that of serum allowed to stand with its clot at room temperature for 24 hours (Table I). The total acid phosphatase increases when the serum is allowed to stand with its clot, due to leakage of enzyme from the red cells even in the absence of any visible haemolysis.

It was found that, in general, the activity is retained for a sufficiently long time to complete the analysis, provided the serum is stored immediately after separation in the refrigerator and there has been no contamination. On the other hand, every minute the separated serum is left at room temperature the *p*H rises and the stability of the enzyme decreases. Sera which are freshly separated and immediately used for enzyme determination, and then left in the refrigerator, usually give slightly lower values on the following day probably due to the time the specimen is left out during the analysis.

TABLE I

STABILITY OF ACID PHOSPHATASE (AT 0° C. AND −15° C. SEPARATED FROM CLOT, AND AT ROOM TEMPERATURE IN CONTACT WITH CLOT, KING-ARMSTRONG ACID PHOSPHATASE UNITS)

Serum No.		Total	Formol-stable	Tartrate-labile
1	Fresh	1·5	1·3	0·3
	24 hr. 0° C.	1·4	1·2	0·4
	24 hr. +20° C.	2·1	1·3	0·4
2	Fresh	2·1	1·6	0·3
	24 hr. 0° C.	1·9	1·7	0·3
	24 hr. +20° C.	2·5	1·7	0·3
3	Fresh	1·6	1·3	0·3
	24 hr. 0° C.	1·6	1·2	0·3
	24 hr. +20° C.	2·0	1·4	0·2
4	Fresh	2·3	1·8	0·3
	24 hr. 0° C.	2·1	1·8	0·1
	24 hr. +20° C.	2·4	1·7	0·3
5	24 hr. 0° C.	2·0	2·0	1·0
	24 hr. +20° C.	2·8	2·4	1·1
6	Fresh	—	96	—
	5 days 0° C.	104	96	98
	5 days −15° C.	102	100	96
	14 ,, 0° C.	—	104	—
	14 ,, −15° C.	—	108	—

Choice of Diluent in Case of High-activity Sera.—With high-activity sera, dilution with saline, normal serum, or very low activity serum gives practically the same value, provided the determination is made immediately after dilution. Some typical results (Table II) also show that there is enzyme inactivation during incubation at 37° C. It is, however, preferable to dilute the serum with the citrate buffer (*p*H 4.9), as the enzyme is more stable at room temperature at this *p*H, and inactivation caused by short delays after making the dilution is thereby reduced. The alternative method of using undiluted high-activity sera for shorter incubation periods is also useful, e.g., the result of a 10 min. hydrolysis multiplied by 6 is taken to be equal to hydrolysis for one hour, i.e., units.

TABLE II

ACID PHOSPHATASE OF DILUTED HIGH-ACTIVITY SERUM (UNITS PER 100 ML. OF UNDILUTED SERUM)

Period of Incubation	Diluent	
	Saline	Serum
0·5 hour	648	644
1·0 ,,	632	600
1·5 ,,	588	560

Short Cuts in Original A.A.P. Method. — The original A.A.P. method (Kind and King, 1954) requires the further addition of three reagents for the development of colour after the enzyme reaction is stopped by the addition of NaOH. Two of these can be mixed for use as a single reagent, viz., the A.A.P. and the bicarbonate, and this gives the same results as the original procedure.

Method for Total and Tartrate-labile Acid Phosphatase

Serum is separated within a few hours of clotting. For best results the estimation should be done immediately. If this is not possible, the serum should be put in the refrigerator immediately after separation and the estimation carried out (preferably) within 24 hours.

Three tubes are prepared (A=total, B=tartrate, C=control) each containing 1 ml. of buffer and 1 ml. of substrate. To tube B is added a drop (0.05 ml.) of M-tartrate (final concentration 0.023 M). The tubes are placed in a water-bath at 37° C., and after about 3 min. 0.1 ml. of serum is accurately measured into tubes A and B at timed intervals (say 30 sec.), mixed well, and the incubation is allowed to proceed for exactly one hour. (Shorter incubation periods are used for high activity sera.) The hydrolysis is stopped by the addition of 1 ml. of 0.5 N-NaOH to A, B, and C, and mixed well. Now 0.1 ml. of serum is added to the control tube C, and 2 ml. of A.A.P. reagent is added to each tube, followed by 1 ml. of ferricyanide. The contents of the tubes are mixed well after each addition.

Standard.—This is made up of 1.1 ml. buffer + 1 ml. of dilute standard phenol solution + 1 ml. 0.5 N-NaOH + 2 ml. A.A.P. reagent + 1 ml. ferricyanide.

Blank.—The blank is the same as the standard, except for water in place of the phenol solution.

The colours develop immediately and are stable for at least one hour if kept in dim light. They are read in a photoelectric colorimeter, with a green light filter (Ilford 624, 510 mμ).

Calculation.—This is as follows:

Total acid phosphatase (in King-Armstrong units per 100 ml.):

$$\frac{A-C}{\text{Standard}-\text{blank}} \times 0.01 \times \frac{100}{0.1}$$

$$= \frac{A-C}{\text{Standard}-\text{blank}} \times 10$$

Tartrate-labile acid phosphatase (in King-Armstrong units per 100 ml.):

$$= \frac{A-B}{\text{Standard}-\text{blank}} \times 10$$

Notes. — For the estimation of tartrate-labile phosphatase alone, the control C is not necessary, but the simultaneous determination of the total acid phosphatase is valuable, and hence the inclusion of the control tube C.

Controls C[1], similar to C but with the addition of a drop of M-tartrate, gave the same reading as C and hence it has not been necessary to include a control for tartrate.

Instead of the addition of a drop of M-tartrate, 1 ml. of substrate and 1 ml. of a buffer prepared by appropriately diluting M-tartrate with citrate buffer to give a final molarity for tartrate of 0.025 could be used for tube B. The former, however, is preferable as it makes the method simpler and more convenient. The dilution caused by the addition of the drop in B is, for practical purposes, almost negligible and does not affect the results beyond the limits of experimental error.

As nearly equal values for A and B are usual in normals and most clinical cases, since the normal tartrate-labile fraction is very low, it is generally preferable to run A and B in duplicate and to repeat doubtful or border-line cases.

Formol-stable acid phosphatase may be included by the addition of a further tube D which is identical with A except for the addition of 0.05 ml. of neutralized 20% formaldehyde, adjusted to *p*H 4.9.

$$\text{Formol-stable acid phosphatase} = \frac{D-C}{\text{Standard}-\text{blank}} \times 10.$$

Results

The total and tartrate-labile acid phosphatases were determined by the above method in a total of 65 normals (43 males and 22 females), the blood being provided from blood donors arranged through the Blood Transfusion Services. Formol-stable acid phosphatase was also determined in 26

TABLE III

TOTAL AND TARTRATE-LABILE SERUM ACID PHOSPHATASE OF NORMAL PERSONS

	No.	Total Acid Phosphatase			Tartrate-labile Acid Phosphatase		
		Mean	S.D.	Range	Mean	S.D.	Range
Males	43	1·80	0·61	1·0–4·0	0·33	0·19	0–0·8
Females	22	2·04	0·51	1·3–3·1	0·35	0·14	0–0·6
Totals	65	1·88	0·59	1·0–4·0	0·335	0·18	0–0·8

King-Armstrong acid phosphatase units.

TABLE IV

TOTAL, FORMOL-STABLE, AND TARTRATE-LABILE SERUM ACID PHOSPHATASE OF NORMAL PERSONS

No.	Total Acid Phosphatase			Formol-stable Acid Phosphatase			Tartrate-labile Acid Phosphatase		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
26	1·71	0·41	1·0–2·5	1·00	0·31	0·5–1·8	0·34	0·23	0–0·8

of these bloods. The mean values, S.D., and range of values are set out in Tables III and IV.

In most prostatic cases the formol-stable acid phosphatase has given a reliable indication as to the diagnosis, i.e., as to whether there is malignancy or not (compare Wray, 1956; King, 1957), but in a few cases the results have been equivocal and in these the tartrate-labile result has agreed with the final diagnosis. Other conditions, such as carcinoma of the breast, in both female and male, have often given results above the normal by both total and formol-stable procedures, but have nearly always yielded figures within the normal range for the tartrate-labile acid phosphatase. A further statement will be given in a future paper; a few representative results are given here (Table V).

TABLE V

NON-PROSTATIC CASES OF ELEVATED TOTAL AND FORMOL-STABLE ACID PHOSPHATASE WITH NORMAL TARTRATE-LABILE PHOSPHATASE

Case, Age, and Sex	Units of Acid Phosphatase per 100 ml. Serum		
	Total	Formol-stable	Tartrate-labile
Carcinoma of breast:			
H. 52 M	15·2	12·3	0
	10·4	6·6	1·2*
O. 72 F	10·3	7·4	0·1
R. 64 F	7·2	4·4	0·1
F. 44 F	12·4	10·4	0·4
L. 58 F	6·0	5·0	0
Carcinoma of bronchus:			
M. 52 F	6·2	4·9	0
Jaundice:			
H. 19 M	5·4	4·6	0·2
B. 28 F	5·2	4·4	0·8*

* Above normal.

Discussion

When modifications of the King-Armstrong procedure (1934) using Folin-Ciocalteu reagent were tried for estimating the tartrate-labile component, it was found necessary to include an additional control tube C^1 for tartrate. This control generally gave slightly higher readings than C, and the tartrate-labile component had to be calculated on the basis $(A-C)-(B-C^1)$. This was emphasized when increasing concentrations of tartrate from 0.012 M upwards were tried, when it was found that the difference between C and C^1 also increased. This means that the tartrate by itself produces a certain amount of colour with the Folin-Ciocalteu reagent. The Fishman and Lerner method (1953) does not appear to take this factor into account. The final tartrate concentration in the Fishman and Lerner method is 0.019 M, but this has been increased to get optimum concentrations for tartrate. On the whole it was found that the use of Folin-Ciocalteu reagent, with the precipitation and separation of proteins which follow, makes the method more tedious and time consuming.

With the A.A.P. reagent, however, the method has been made much easier and simpler. The procedure has also been shortened by mixing the A.A.P. and bicarbonate before addition.

The p-nitrophenyl phosphate method (Ohmori, 1937; King and Delory, 1939; Bessey, Lowry and Brock, 1946) has also been tried to see how far it is advantageous. It was found that though the rate of hydrolysis with p-nitrophenyl phosphate is slightly faster at the acid *p*H, yet this is not appreciable enough to cut down the incubation time. The method requires no further addition of reagent beyond that of NaOH to stop the enzyme reaction, but the yellow colour produced is an unsuitable colour for ordinary photoelectric colorimetry. A quartz spectrophotometer ("unicam" SP 500) had to be used for the readings, and much of the time saved on the addition of reagents was spent in manipulating this instrument.

It was found that with the formaldehyde method the colour produced with A.A.P. is not so stable as it is with the total or tartrate-labile acid phosphatase. The colour increases slightly with time in the presence of formaldehyde and attains a somewhat different shade of rose-pink than those without formaldehyde at the end of about half an hour. The error, however, was negligible if a control tube was run with formaldehyde and the readings taken within 30 min.

The formol-stable fraction, however, is not such a sensitive index for prostatic acid phosphatase as

the tartrate-labile component. Several cases have been encountered, especially in carcinomas other than of the prostate, where the formol-stable fraction was raised in the presence of a normal tartrate-labile component (Table V). We are also assessing the value of the tartrate-labile component as determined by the above method in elucidating doubtful or border-line cases of prostatic carcinoma, especially those in which a normal formol-stable acid phosphatase is obtained.

Our experience on the stability of serum acid phosphatase is in agreement with the findings of Daniel (1954). We do not, however, favour the idea of allowing the serum to stand with the clot, as this increases the total acid phosphatase due to leakage of the enzyme from the red cells (Table I). Davison (1953) found that a serum from a case of metastatic carcinoma of the prostate retained its high activity for periods up to 112 days in the deep-freeze (−15° C.). We find that serum acid phosphatase is equally stable at 0° C. for periods up to 14 days, provided the serum is put in the refrigerator immediately after separation (Table I).

As regards the normal range of tartrate-labile or prostatic acid phosphatase (Table III), in a total of 65 apparently healthy adult subjects (43 males and 22 females) only one male gave a value as high as 0.8 units, two males 0.7 units, and one male and two females 0.6 units. All the rest gave values below 0.6 units. We would fix 0.7 units as the upper limit of normal for prostatic acid phosphatase. This is two standard deviations (0.18) from the mean (0.335), and is 0.1 unit higher than that proposed by Fishman *et al.* (1956) and by Davis and Ward (1956), and is in accordance with the value proposed by Nobles *et al.* (1957). The distribution for prostatic acid phosphatase was negatively skewed. We would consider prostatic conditions which give values in excess of 0.7 units as suspicious.

Of the total acid phosphatase values, there were only two above 3 units (one male 4 units and one female 3.1 units) and of the 26 cases where formol-stable acid phosphatase was also done (Table IV), none above 1.8 units. There had been a trace of haemolysis in some of the specimens, and this may account for the slightly higher mean for the total acid phosphatase for females. We do not think that there is any significant difference in the mean values for males and females whether for total acid phosphatase or for the tartrate-labile component. As the mean values for tartrate-labile acid phosphatase are practically the same for both males and females it is probable that this "prostatic" component in normal persons arises from sites other than the prostate. Haemolysis does not affect the tartrate-labile acid phosphatase, as the red cell enzyme is stable to tartrate.

Summary

A simple method for the determination of tartrate-labile acid phosphatase using phenyl phosphate and an amino-antipyrine reagent is described. It is shown that the optimum concentration of tartrate necessary is about 0.025 M in the buffer-substrate-enzyme mixture for the complete inhibition of the prostatic component in blood serum. It is also shown that serum acid phosphatase is unstable at room temperatures but stable at 0° C.

The total and tartrate-labile acid phosphatases have been determined in 65 normal persons, and the formol-stable acid phosphatase in 26 of them. An upper limit of 0.7 King-Armstrong units is proposed for the tartrate-labile acid phosphatase in normal subjects.

We should like to thank Dr. Constance Wood, Dr. G. F. Joplin, Dr. P. L. Mollison, and Dr. P. E. Booth for some of the specimens. One of us (K. A. J.) is grateful to the Governments of the United Kingdom and Ceylon for a Colombo Plan Fellowship under which this work was done.

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TECHNICAL METHODS

A Micro Method for Detecting Indol Formation

NICHOLAS KOVACS

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(RECEIVED FOR PUBLICATION JUNE 14, 1958)

The acid in the commonly used indol reagent described by Kovacs (1928, 1932) and *Diagnostic Procedures and Reagents* (1950) changes the reaction of the medium in which the indol production is tested and prevents the further observation of biochemical processes, killing the bacteria themselves.

It was found that, by heating 0.1 ml. or less of culture (broth or peptone water), indol production by enteric bacteria could be easily detected. Four capillary drops of the fluid medium to be tested are put into a test tube of about 12 by 75 mm. and 4 drops of the indol reagent are added from a dropping bottle. The mixture is shaken lightly and if it does not turn red immediately it should be heated gently for a moment, without actual boiling, in the flame of a Bunsen burner. Heating doubles the sensitivity of the reaction seen at room temperature.

For routine work the culture fluid which is to be tested is collected with a spiral-ended wire. This is a simplified version of the one used by Takátsy (1955) for virus work and of the volumetric platinum cylinder described by Berridge (1954). Both are manufactured. The cylindrical spiral can be easily made in the laboratory from a 22 gauge platinum wire by turning eight coils around a 1 in. by 5/32 in. Whitworth metal thread steel screw (about 4 mm. diameter). When preparing the spiral $\frac{1}{2}$ in. (1 cm.) of wire is left straight at the commencement. When the spiral has been wound, this short end is bent back with forceps into the centre so as to form an axis (Fig. 1). This axis enables the spiral loop to hold the contents more easily. The coils are now pressed together with the forceps so that they are tightly closed. The spiral loop contains about 0.08 to 0.09 ml. of water.

The use of spiral wires dispenses with Pasteur pipettes and saves considerable time and material.

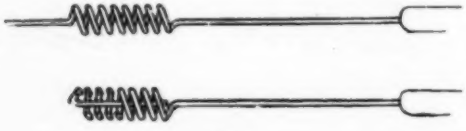

FIG. 1

Method

Four drops of the indol reagent are filled from a dropping bottle into a Kahn tube. The flame-sterilized spiral wire is loaded with the culture fluid to be tested for indol, and, while holding the tube almost horizontally, the contents of the wire are washed off by rotating the spiral in the reagent. When the indol reaction is not immediately apparent, the tube is heated carefully over the Bunsen burner for one or two seconds only and the reaction observed. In serial examinations, when many cultures are tested in succession for indol formation, the indol reagent is added to a series of Kahn tubes and two or three spiral wires are used alternately as they take much longer to cool than ordinary loops.

The micro-method of indol determination with platinum spiral wire and heat proved a useful and simple test, especially in mass examination of cultures from faeces, egg-pulp, Moore swabs, etc.

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Correction

We regret that there is an error in the paper, "The Antibody Content of Single Cells" (November, 1958, vol. 11, No. 6, p. 544, column 1, line 14). Instead of "an average of 7.8 grains in stripping film," it should read "an average of 1.8 grains in stripping film."

The Estimation of Protein in the Cerebrospinal Fluid Using the M.R.C. Photometer

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(RECEIVED FOR PUBLICATION MARCH 28, 1958)

In the communication by Yeoman (1955) it is stated that the proportion of cerebrospinal fluid to 3% aqueous salicylsulphonic acid is one to three when used to determine the protein content in the M.R.C. photometer. This method has been studied by comparing results using his method and results obtained by the micro-Kjeldahl method (King, 1951) on the same specimens. Twenty specimens were examined in which the levels were representative of all levels found in practice, i.e., normal, moderate increase, and marked increase. The results showed that the M.R.C. photometer method was fairly accurate if 1 ml. of cerebrospinal fluid was added to 4 ml. (not 3 ml.) of 3% salicylsulphonic acid. One of us (J. G. A.) communicated with Dr. Yeoman and he agreed that the proportions should have been one to four and not one to three. It was found, however, that the true protein level (x) was slightly higher than the reading on the photometer (y), the two being related by the equation $x = \frac{16y + 20}{15}$

When the protein concentration exceeds 120 mg./100 ml. it is necessary to dilute the cerebrospinal fluid with water because the curve is no longer linear above this value. The use of saline as a diluent gives increased readings.

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The "Normal Range" of the Prothrombin Time Estimated by Quick's Method

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(RECEIVED FOR PUBLICATION JUNE 26, 1958)

Armitage and Ingram (1958) have recently investigated some errors affecting Quick's prothrombin time. They found that the differences between the prothrombin times of normal persons were much greater than could be explained by the experimental error of the method, and that it was possible to devise a simple rule of thumb to express the amount by which a given "control" plasma might be expected to differ from another normal plasma.

Prothrombin times were measured on 56 differing pairs of normal plasmas, with four replicate readings from each sample. The standard deviation of the difference between mean times for the paired subjects was about 9% of the average of the prothrombin times of the pair. Experimental error would have contributed only some 2% to this figure, or rather less than one-quarter of the total standard deviation. A reduction in the number of replicate readings made on the two samples will appreciably increase the contribution of experimental error, but will not greatly increase the total standard deviation, which can therefore be taken to be about 9% of the average prothrombin time, irrespective of the number of readings. Had only two replicate readings been obtained instead of four, the error contribution would have risen only to 3% of the average prothrombin time, or one-third of the total standard deviation, in the above experiments.

This means, for example, that in 95 cases out of 100 two normal prothrombin times will differ by less than about $2 \times 9\%$, or say *one-fifth* of their average (the rule is expressed in this way to be independent of the activity of the brain extract). In the control of anticoagulants, such small differences are clearly of no importance, but in the study of bleeding disorders and as a precautionary investigation, e.g., before needle biopsy of viscera, it may on occasion be useful to test borderline cases in this way.

It can usually be assumed that the patient's prothrombin time will differ materially from the control, if at all, only in one direction, e.g., it is often of no consequence if the patient's time is shorter than the control time. In this situation it may be said that only about once in 40 times, i.e., 2.5 times out of 100, should a normal prothrombin time exceed the control

TABLE FOR DETERMINING THE NORMAL RANGE OF THE PROTHROMBIN TIME

Frequency with which Tabulated Time would be Exceeded in Tests on Paired Plasmas		Control Prothrombin Time (sec.)															
Two-directional Comparison	One-directional Comparison	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 in 20 (10 in 200)	1 in 40 (5 in 200)	12	13	14	16	17	18	19	20	22	23	24	25	26	28	29	30
1 in 100 (2 in 200)	1 in 200	13	14	15	17	18	19	20	22	23	24	25	27	28	29	31	32

by more than one-fifth of the mean of both times. This type of comparison we may call "one-directional."

An observed difference between a patient's and a control prothrombin time would thus be significant at the 5% level (in a two-directional test) or the 2½% level (in a one-directional test) if the difference exceeded 0.18 times the average of the two readings, and at the 1% level (two-directional) or 0.5% level (one-directional) if it exceeded 0.23 times the average.

Put another way, this means that, if a patient's prothrombin time exceeds 1.20 times the control reading, this is a difference which would be exceeded only once in 40 times (in that direction) or once in 20 times (in either direction) in comparisons between a normal control and another normal individual; and that, if the patient's time exceeds 1.27 times the control reading, this difference would be exceeded only once in 200 times (in that direction) or once in 100 times (in either direction).

For facility, these multiples have been rounded off to 1.2 and 1.3 respectively and the values obtained which correspond to various control prothrombin times between 10 and 25 sec. have been tabulated. If the test plasma exceeds either of the given values corresponding to the observed control, then it may be considered abnormal with the degree of probability attached.

Method

Select the column corresponding to the observed control time: then, in a series of 200 comparisons between paired plasmas from a control and another normal subject, five subjects would be expected to give prothrombin times longer than the value given in the middle row, but only one longer than the value in the bottom row. If two-directional comparisons are required the shorter time should be entered along the top (control) line, and the longer time compared with the tabulated entry.

The tabulated entries are rounded to the nearest second. More accuracy and intermediate values may be obtained by multiplying the observed control time by 1.20 and 1.27 for the one-directional 1 in 40 and 1 in 200 risks, respectively.

Example: Control readings, 17.2, 17.6 sec., mean 17.4 sec. Longest normal reading expected at 1 in 40 risk (one-directional):

(*a*) from Table; *col. headed* 17 *sec.*, 5% reading, 20 sec.

(*b*) by calculation; $17.4 \times 1.20 = 20.9$ sec.

Patient's readings, 20.8, 21.4 sec., mean 21.1 sec. At these risks this should be considered just abnormal.

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A Substitute for the Feulgen Staining Technique

H. B. W. GREIG

From the Department of Haematology, South African Institute for Medical Research, Johannesburg, South Africa

(RECEIVED FOR PUBLICATION JUNE 31, 1958)

The Feulgen "nucleal" reaction as applied cytologically (Feulgen and Rossenbeck, 1924) is based on Feulgen's finding that among the products of mild acid hydrolysis of nucleoprotein is a substance which gives a positive Schiff's test (recoloration of leucobasic fuchsin) for aldehydes. The Feulgen reaction is probably specific for deoxyribonucleoprotein (Pearse, 1953; Kurnick, 1955); thus nuclear chromatin and chromosomes are stained. Nucleoli and cytoplasm, which contain ribonucleoprotein, are Feulgen negative.

The use of the Feulgen stain in haematological material has been described by La Cour (1944), Pittaluga and Bessis (1944), Gardikas and Israëls (1948), Rheingold and Wislocki (1948), Thoma (1950), and Marinone (1951). The method has been used principally for the localization of deoxyribonucleoprotein and in the study of chromosomes and the processes of cell mitosis. Besides these rather academic uses, the stain has a practical use, that of assisting in the identification of primitive cells by virtue of the clear demonstration of the nucleoli (Hayhoe and Whitby, 1955).

Some years ago, when the author first used the Feulgen stain, he attempted to counterstain blood and bone marrow films with Leishman's stain, but found that stain diluted in the ordinary way with buffered distilled water approximately 1 in 1 to 1 in 2, with the customary staining time of five to 10 minutes, gave useless results on account of the intensity of uptake of the Romanowsky stain by the nuclear chromatin. It was necessary to dilute the Leishman stain about 1 in 10 to 1 in 20, and to reduce the staining period to about one minute. This resulted in the Feulgen-positive material taking up a deep basophilic colour, while the cytoplasm and nucleoli remained unstained.

The Feulgen staining technique is a fickle one. Peter Gray (1954) says of the Feulgen reaction: "Its nuclear reactions appear to depend upon the selective staining of the thymonucleic acid, but the stain itself is so difficult and so temperamental that it is doubtful that it has occasioned any real advance in microtomy."

Schiff's reagent does not keep well (Lhotka and Davenport, 1949; Lillie, 1951). The staining of the nuclei of blood cells is often faint and detail difficult to discern. On account of the improvement in contrast and definition of the Feulgen positive material, the author continued to use diluted Leishman's or Wright's stain after Feulgen staining.

Subsequently, the effect of diluted Wright's or Leishman's stain was tested on smears fixed in the usual way and hydrolysed for 15 minutes at 56° C. in normal hydrochloric acid omitting the treatment with Schiff's reagent. It was found that this gave similar results to those obtained with diluted Wright's or Leishman's stain after using Schiff's reagent. The technique is easy and the reagents used are immediately available in any haematological laboratory.

Method

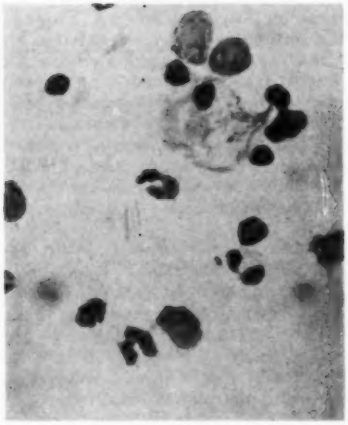
Air-dried smears of blood or bone marrow are fixed for three minutes in absolute methanol, or, better, in absolute methanol containing 10% formalin (4% formaldehyde solution). After fixation, the slides are rinsed well with distilled water and then immersed in normal hydrochloric acid at a temperature of 56° C. and left for 15 minutes. They are rinsed with distilled water and then stained for approximately 30 to 60 seconds with a Romanowsky stain, e.g., Wright's or Leishman's, diluted 1 in 10 with buffered distilled water (*p*H 6.8). The slide is then rinsed with distilled water and dried in the air. Fig. 1 shows examples of blood and marrow films stained by this technique. Chromatin and chromosomes are stained a deep blue-black. Nucleoli appear very clearly as "holes" in the nuclei; cytoplasm is not stained, but azuriphil granules and eosinophilic granules often stain with the eosin component of the Romanowsky stain.

Discussion

The principal use of this stain (and the Feulgen stain) in diagnostic haematology lies in the demonstration of the presence, size, and number of nucleoli in nuclei. The value of this is greatest in leukaemia, especially in cases of aleukaemic acute leukaemia with "microblasts." These are scanty on the blood films and resemble small lymphocytes when stained by the Romanowsky stains. The use of this stain to demonstrate clearly the presence of nucleoli has been greatly appreciated by technicians.

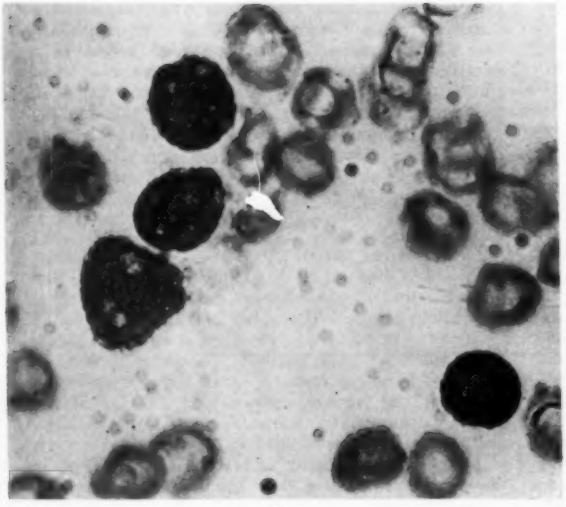
Although this staining technique appears to stain the same structures as the Feulgen stain does, no claim is made for it as a specific cytochemical procedure for deoxyribonucleoprotein. Further study is required on this point and on the rationale of the procedure.

Flax and Pollister (1949) and Pollister, Himes, and Ornstein (1951) showed that the dye azure A stained the nucleoproteins by virtue of their phosphoric acid groups, but Pollister did not use azure A after acid hydrolysis to differentiate deoxyribonucleoprotein from ribonucleoprotein as the technique described appears to do. The mild acid hydrolysis probably "uncovers" the phosphoric acid groups of deoxyribonucleoprotein, while differentiation probably depends on the greater

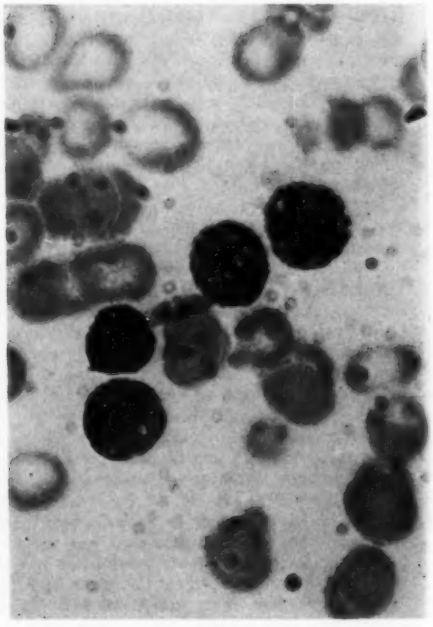


(*a*)

(*b*)



(*c*)



(*d*)

FIG. 1.—(*a*) A low-power (× 480) view of the bone marrow from a case of very early acute leukaemia, showing a marked increase in the number of "blast" cells although normal cells of both the myeloid and erythroid series are present. The peripheral blood showed a leucopenia. (*b*) A high-power view (× 1,200) of two of the "blasts" from the same marrow. (*c*) and (*d*) "Blasts" in blood films of two different cases of acute leukaemia (× 1,200).

sensitivity of ribonucleic acid to acid hydrolysis (Kurnick, 1955) and the fact that ribonucleic acid is very easily removed from the cell by such treatment (Vendrely-Randavel, 1949).

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The "Lambeth" Technitray

O. A. N. HUSAIN

From the Group Laboratory, Lambeth Hospital, London

(RECEIVED FOR PUBLICATION DECEMBER 9, 1958

The biggest obstacle to the design of an all-purpose technician's carrying unit has always been that of the widely differing requirements of individual laboratories. An attempt to overcome it has been made by the construction of a basic tray with a drawer beneath and an extensive range of interchangeable inserts.

The final design is a compromise between the numerous ideas from this and other laboratories and the limiting factors of construction and costs.

Made throughout of thin, polished aluminium, the whole unit, with the standard set of components as illustrated (Fig. 1), measures 14×8×5 in. with the handle 10 in. high, and weighs under 4 lb. Being metal, it is easily cleaned and sterilized and a separate slip-on plastic cover allows it to be carried in inclement weather.

The drawer below, 2 in. deep, can be used for syringes or notes, or be fitted with further inserts. The tray, as shown, provides for 24 red or white blood cell bulb type pipettes (in two layers), 18 bijou bottles, 22 Kahn tubes, five "universal" containers,

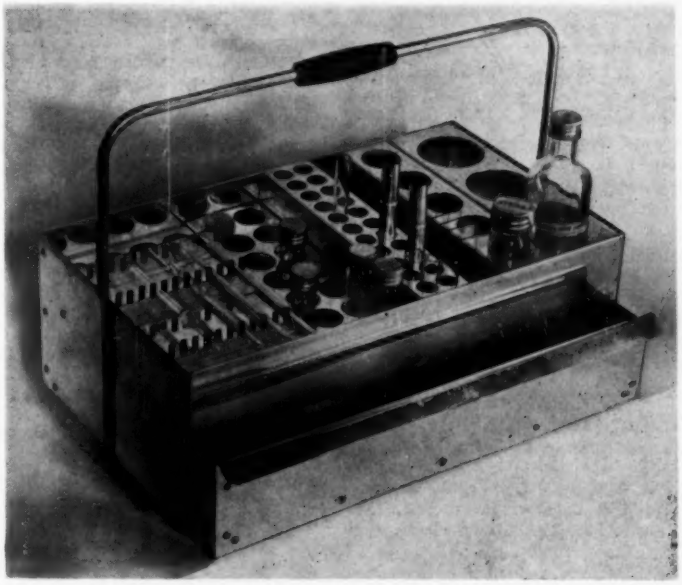


FIG. 1.—The Lambeth "technitray."

three blood culture bottles, and a rack of 40 slides (or 80 back to back).

Additional inserts are made to take the standard $\frac{5}{8}$ in. test tubes, the $\frac{1}{2}$ in. tubes, a single row of bijou bottles, and for those no longer using the bulb pipette there are simple trough inserts to take the straight-sided 20 and 50 c.mm. pipettes.

Moreover, the firm producing the unit is prepared to manufacture, quite cheaply, additional inserts to special designs. In fact the most controversial rack, that for cotton-wool, spirit, and prickers, has already been made in four different patterns. A gap of any size can be created by simple trough inserts or by small screw clamps on the tray sides.

Designed primarily for the clinical laboratory worker, it may also be of use in other branches of the laboratory world.

The " technitray " has been manufactured for us by Messrs. Luckham Ltd., of 591, Kingston Road, Raynes Park, London, S.W.20.

The Protides of the Biological Fluids

The 7th colloquium will be held at St. John's Hospital, Bruges, on May 1–3, 1959.

1. Special Topics:
   - (1) Nutritive value of protein and protein subnutrition
   - (2) Biosynthesis of proteins
   - (3) The role and metabolism of carnitine
   - (4) Foetal protein
2. General Topics:
   - A. Biochemical:
     - (1) Amino-acids
     - (2) Polypeptides
     - (3) Proteins: lipo-, glyco-, and heteroproteins. Proteins with specific activity in connexion with coagulation, immunity, hormonal activity
   - B. Medical:
     Diagnosis in blood, urine, C.S.F.
     Therapy and nutrition
   - C. Technical:
     - (1) Chemical methods such as chromatography, polarography, electrophoresis
     - (2) Microbiological methods

Round-table Subjects:

- (1) Technical advances in the analysis of proteins and related substances
- (2) Advances in the pathology of proteins, polypeptides, and amino-acids

For further information apply to Dr. Peters, Bruges.

Seventh Congress of the International Society of Haematology

The International Society of Haematology held its seventh congress at the Palace of Congresses in Rome from September 7 to 13, 1958, under the presidency of Professor GIOVANNI DI GUGLIELMO.

The main topics included immuno-haematology, the haemorrhagic diseases, leukaemia, the spleen and the reticulo-endothelial system, anaemia, isotopes in haematology, vitamin B_{12}, anticoagulants, haemophilia, blood and blood-forming cell cultures, haematological genetics, and paediatric haematology.

Immuno-haematology

Some of the modern views on immuno-haematology and on acquired or auto-immune haemolytic anaemia were outlined by J. H. JANDL (Boston, U.S.A.). These anaemias are characterized by the adsorption on to the red cell surface of substances of a protein nature diminishing the suspension stability of red cells. Antigen-antibody complexes adhere to the red cell envelope and cause direct agglutination by antiglobulin serum. Sensitized red cells are filtered by the spleen and agglutinated red cells by the liver. J. DAUSSET (Paris) reviewed antileucocytic auto-immunization, which can be allergic or spontaneous. Allergic antibodies are usually antinuclear, as in disseminated lupus erythematosus, and they can be estimated by complement-fixation and leuco-precipitation tests. W. J. HARRINGTON (St. Louis, U.S.A.), describing immune reactions of platelets, stated that the lack of suitable serological procedures hampered progress in this field. The platelet has a complex antigenic structure and is highly susceptible to auto-immune reactions. Antibodies against platelet antigens can be natural or immune, complete or incomplete, of auto- or iso-type, or agglutinins or lysins. Steroid therapy inhibits antibody production and splenectomy removes the site of antibody production. H. E. WILSON (Ohio, U.S.A.) used rabbit antisera against human leucocytes to test leucocytic agglutinins, particularly after blood transfusions. It was found that these leucocytic agglutinins are predominantly in the γ globulin range and are inhibited by the albumin fraction. R. T. SILVER (Maryland, U.S.A.) found that the overall antibody response of leukaemic patients against typhoid, mumps, influenza, diphtheria, and tetanus antigens was less than in normal controls.

Haemorrhagic Diseases

Christmas factor and Hagemann factor mutually correct each other, as observed by J. P. SOULIER (Paris, France). In haemophilia, said F. KOLLER (Zurich, Switzerland), in one and the same family only mild or only severe cases are found. J. B. GRAHAM (U.S.A.) found that mothers of patients with

Christmas disease showed few definite signs of haemorrhagic disorder. A. A. SHARP (Oxford, England) discussed the breakdown of the normal haemostatic mechanism associated with the lack of normal blood fibrinogen as a complication of pregnancy.

J. HUGUES (Liège, Belgium) observed that certain antihistamine drugs inhibit proper clot formation. H. D. WALLER (Marburg, Germany) showed that the sources of energy for platelets are derived from glycolysis. W. LEHMANN (Kiel, Germany) re-examined some of the patients in the Aaland islands with Willebrand-Jürgens' thrombopathy and found that many are deficient in anti-haemophilic globulin. M. M. WINTROBE (U.S.A.) found that 11 cases of a series of 66 with idiopathic thrombocytopenic purpura were of the self-limiting type and did not require treatment. In chronic cases splenectomy is the only practical measure currently available; steroid therapy has a very limited application. J. SABLINSKI (Warsaw, Poland) said that among a series of 111 haemophilic patients, 42 were sporadic cases. In Poland the incidence of haemophilia is 1 in 50,000. H. A. WURZEL (U.S.A.) examined the coagulation defects in 102 patients with uraemia, and showed some defect, but other factors play a major part in this bleeding tendency.

Leukaemia

The myeloproliferative syndromes, with particular reference to Di Guglielmo's disease, were the subject of the Adolfo Ferrata lecture given by W. DAMESHEK (U.S.A.), who divided this disease into three stages, e.g., "erythraemia," that is, erythroblastosis, "erythroleukaemia," that is, erythroblastosis and myeloid hyperplasia, and myeloblastic leukaemia. He suggested that the anaemia was due to ineffective erythropoiesis and possibly to some haemolytic component as a result of haem diversion. E. NEUMARK (London, England) described the haematological and morbid anatomical changes in a series of seven cases of erythraemic myelosis. The infiltration in liver and spleen and other organs differs from that seen in myeloid leukaemia. Most of the cells in the marrow and other tissues are not megaloblasts, but abnormal erythroblasts. J. H. BURCHENAL (U.S.A.), who used purine antagonists, folic acid antagonists, and steroids in a group of 253 children with acute leukaemia, reported that the average survival had been increased to over a year. M. M. WINTROBE (U.S.A.), in an Adolfo Ferrata lecture on the changing aspect of clinical investigation illustrated by a study of acute leukaemia, said that pharmacological advances had increased the survival rate of patients with acute leukaemia, but efforts in myeloblastic leukaemia had failed almost completely. F. H. BETHELL (U.S.A.) reviewed 500 cases of leukaemia in adults and found that during recent years the myelomonocytic (Naegeli) variant of acute myeloid leukaemia had increased most. Symposia on the aetiology and chemotherapy of leukaemia were held.

The Spleen and the Reticulo-endothelial System

Increased susceptibility to infection after splenectomy was studied by C. A. DOAN (Columbus, U.S.A.), who used a Foshay-killed tularaemia vaccine. He found hypogammaglobulinaemia and atypical globulin patterns in some patients, but he concluded that the underlying disease was much more important than the presence or absence of the spleen for the development of specific antibodies. F. REIMANN (Istanbul, Turkey) examined patients with portal hypertension haematologically and by a catheter during operation. The splenic material contained two to three times the number of neutrophils than the blood, perhaps due to their retention in the spleen.

Anaemia

No major abnormality of globin synthesis was found in thalassaemia by C. V. MOORE (U.S.A.), who investigated the biosynthesis by the incorporation of tagged protoporphyrin into haem. M. BALDINI (U.S.A.) in an investigation of erythrokinetic methods in various blood dyscrasias concluded that the determination of the faecal urobilinogen was of little value. M. BESSIS (Paris, France) followed the iron metabolism by the use of the electron microscope. When red cells are ingested by reticular cells, molecules of ferritin can just be appreciated in groups of four little dots by the electron microscope. M. POLLYCOVE (U.S.A.) examined cases with refractory anaemia and erythroid hyperplasia by following the fate of plasma-bound radio-iron. Erythropoiesis in pernicious anaemia, thalassaemia, and Di Guglielmo's disease, though hyperplastic, is largely ineffective because of erythron destruction in the marrow. F. RENDA (Ankara, Turkey) found that iron given parenterally to women in the last three months of pregnancy complicated by iron-deficiency anaemia did not benefit the foetus.

Isotopes in Haematology

G. B. JERZY GLASS (New York, U.S.A.) found that following the administration of vitamin B_{12} containing Co^{60} the maximal uptake of radioactivity by the liver was seven to 20 days after the start of the treatment and remained at that level for some time. The storage of radioactivity in the liver remained constant for a long time and only decreased by 1–10% per month. The deposition of radioactive vitamin B_{12} was reduced in a third of a series of patients with liver disease and abolished in a few cases. This may explain the high vitamin B_{12} blood levels in some cases of liver disease. K. A. AAS (Oslo, Norway) found that Cr^{51} tagged normal platelets when transfused to patients with idiopathic thrombocytopenic purpura only survive a day or less compared with the normal of nine to 11 days.

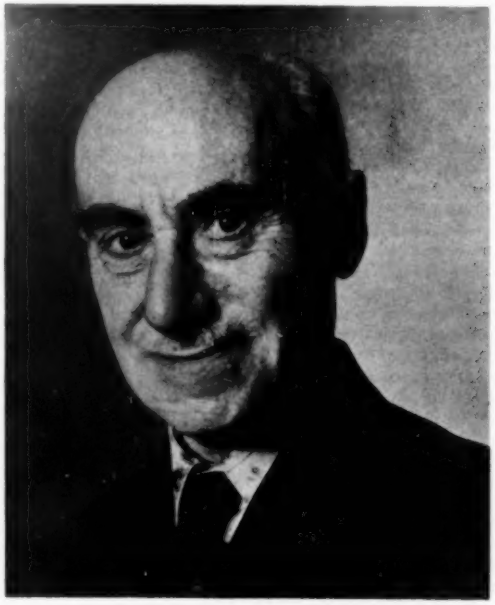
E. NEUMARK.

J. clin. Path. (1959), **12**, 98.

OBITUARY

DAVID NUNES NABARRO

David Nunes Nabarro, who was the first Director of the Pathological Department of The Hospital for Sick Children, Great Ormond Street, London, died at his home in London on October 3, 1958. He was 84 years of age. He was educated at Owens School and University College Hospital, London, and qualified first with a B.Sc at the age of 19, and with the Conjoint Diploma at 23. He passed his M.B., London, examination in 1898, and the M.D. (winning the gold medal) in 1899. He acquired the D.P.H. in 1901, was admitted a Member of the College of Physicians in the same year, and was elected a Fellow in 1917. He had been an exhibitioner and had won numerous prizes and awards during his student career. After holding house appointments in University College Hospital he was appointed to his first pathological post as Assistant Professor of Pathology and lecturer on bacteriology at University College in 1899, and held this appointment, together with that of pathologist to the Evelina Hospital for Sick Children, till 1910. After a brief stay in the provinces as pathologist to the Wakefield Asylum for two years he returned to London in 1912 as clinical pathologist to The Hospital for Sick Children. His whole future was bound up with the pathology of children's diseases, and he had immense attachment to and pride in the development of the department and the hospital which he so dearly loved. He saw them both grow to their present pre-eminence with pride.



In his earlier years he was a member of the Royal Society's Commission which went to Uganda in 1903 to investigate sleeping sickness. He was the joint discoverer with Dr. David Bruce and Dr. Aldo Castellani of the trypanosome and its mode of transmission, but felt that his contribution to the discovery had never been properly acknowledged. Since his days in Wakefield he had acquired an interest in syphilis and dysentery, and these two subjects he studied continuously and with much fervour. He started a clinic at Great Ormond Street for the investigation and treatment of congenital syphilis and juvenile gonorrhoea, and his clinical control of the patients, both as out-patients and as in-patients in his tiny isolated ward, together with the laboratory control, provided an early example of clinical pathology at its best. He loved the children, however crippled, and spent countless hours on even hopeless cases of juvenile G.P.I. He collected his data most meticulously, and after a number of years was persuaded that he had a unique experience of congenital syphilis and should publish it. However, as new cases occurred he would again and repeatedly go right back and alter his tables and data, until eventually in retirement he really got down to the analysis of the data. His book was finally published when he was 80, and will remain the classical British work on the subject, which will be so valuable to those who will now only see very rare cases of congenital syphilis. His work on the dysenteries, especially Sonne dysentery, if it did not reach such fruition at least showed the importance of strict attention to nursing details when children of varying ages are herded together under ward conditions.

His greatest contribution to pathology, however, lies in his early vision of the role of the pathologist and the laboratories in a hospital community. It was no accident that he was a founder member of the Association of Clinical Pathologists when most of the members were from the provinces and the Association was rather frowned upon by the London and academic pathologists, and he was honoured by becoming its President in 1947.

As a person he was quiet and rather mild, except on the rare occasions when he was roused by the stupidities of eminent people. He was at times self-

effacing to a degree, and at other times he would fight fiercely for a cause which he felt was important to medicine and especially to pathology. He was a careful and informed reader of English literature, and his writing showed his meticulous command of English—he was equally critical of other people's grammatical errors. He was an enthusiastic philatelist and a Fellow of the Royal Philatelic Society, and an acknowledged authority on the early stamps of the Falkland Islands. He had a quick sense of humour which was wonderfully devoid of malice. His personality really blossomed, however, in his home. His devotion to his wife and his adoration of his children were patent to all who sat at his table, and his pride when his son joined him as a Fellow of the College of Physicians was touching.

He will always be remembered at Great Ormond Street and by all those who worked with him for his devotion to pathology and his kindliness to his most junior colleagues.

A. GORDON SIGNY.

BOOK REVIEWS

Ciba Foundation Colloquia on Endocrinology, Vol. 12: *Hormone Production in Endocrine Tumours.* Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. 295+Cumulative Index Vols. 1–12; 60 figures. 48s.) London: J. and A. Churchill. 1958.

This colloquium brought together experts and active workers in many fields of endocrinology. The emphasis was on the pituitary-adrenal axis and on the gonads, and particularly on experimental work in animals.

There are two papers on thyroid tumours, one dealing with their experimental induction by thiouracil and the other describing the discovery of a new iodinated protein in human serum. (In the discussion the existence of a goitrogenic human baby food was mentioned, but not identified.)

In a review on serum insulin the authors claimed that they often found high concentrations in patients with islet-cell tumours, but emphasized methodological difficulties. Van Euler reviewed, briefly and thoroughly, present knowledge and recent advances concerning the chromaffine system. Two interesting short papers on human gonadotrophin excretion seem rather out of place.

It is not possible to summarize all the papers on the main theme in a brief review, but certain conclusions can be drawn. As usual, as much is gained from reading the discussions as from the formal papers. Methods of assay and identification, of pituitary hormones much more than steroid hormones, are still very imperfect, and improvements in technique are necessary in many fields before advances can be made. At present, hormone assays are of very little use in the diagnosis of human tumours, except for thyroid and chromaffine tumours. The nature of hormone dependency is uncertain, and there is still controversy (taking a human tumour as an example) whether hormone-dependent carcinoma of the breast is oestrogen-dependent primarily, or only secondarily because oestrogens stimulate the secretion of mammotrophin.

The volume should be valuable reading for experimental pathologists and for endocrinologists, and will keep them informed and stimulated in this important and difficult field. The reviewer found the section by Furth and Clifton on their experimental induction of four different types of monomorphous pituitary tumours particularly interesting. The paper by Dorfman summarizing pathways of adrenal corticosteroid and androgen metabolism is not wholly new, but is valuable nevertheless: in his fig. 2 he implies that A.C.T.H. is active in the pathway of aldosterone synthesis—is this deliberate ?

The cumulative index to the first 12 volumes of these Colloquia is included which will be extremely

useful to all who consult these volumes, namely all endocrinologists. The book is well printed and produced, but 48s. does seem expensive.

D. N. BARON.

Chemistry and Biology of Mucopolysaccharides. A Ciba Foundation Symposium edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii + 323 ; 48 illustrations. 45s.) London: J. and A. Churchill. 1958.

The mucopolysaccharides may be defined as amino-acid carbohydrate complexes with big molecules. They occur in the body in a variety of associations—in cartilage, in connective tissue, associated with blood group specificity, in mucus, in relation to the glycoproteins of plasma, in urine, and so forth. Some occur in micro-organisms and viruses. Our knowledge of mucopolysaccharides is still scanty, and therefore the present volume is largely concerned with the results of work on individual mucopolysaccharides, much of it analytical ; a broad picture of these substances is not yet possible. For the clinical pathologist who seeks to know something about these substances, which are becoming daily of greater biological importance, this book will make interesting and difficult reading. Naturally the different contributions are of varying interest and value. It is impossible not to feel that this published Ciba Symposium, in common with the others, would benefit by drastic editing of the discussions, in which important points emerge ; they would emerge more clearly if the discussions were pruned.

ARTHUR JORDAN.

Tuberculosis in White and Negro Children, Vol I: *The Roentgenologic Aspects of the Harriet Lane Study*. By Janet B. Hardy. (Pp. vii + 122 ; illustrated. 60s.) Vol. II: *The Epidemiologic Aspects of the Harriet Lane Study*. By Miriam E. Brailey. (Pp. 103 ; 8 graphs. 36s.) Harvard University Press (London: Oxford University Press). 1958.

All medical books are out of date by the time they are published, but some more so than others. This is particularly the case with books on tuberculosis.

Here we have two beautifully produced volumes, the result of many years' study, which, by virtue of the rapid advance in the treatment of tuberculosis, have been rendered almost of historical interest.

The first volume consists of an atlas of radiological appearances of the chest and other regions in childhood tuberculosis. This probably represents one of the best collections of such radiographs available. The plates are beautifully reproduced, and their value is enhanced by the photographs of necropsy specimens which accompany some of them. Unfortunately the results of treatment are only available for a short period extending into the streptomycin era. Nevertheless the photographs are most informative, and their perusal would be of great value to all who are working in this field. There is also a discussion on bronchography and bronchoscopy in tuberculosis, and attention is rightly drawn to the important effects of tuberculosis on the bronchial tree. It is rather surprising, however, to find that these techniques are used in children from the age of 4 upwards under local anaesthesia. These procedures are often tolerated badly by adults, and the effect on young children scarcely bears contemplation.

The second part deals with epidemiology and prognosis. An extremely full analysis of these aspects is provided, both in text and tabular form, and as a document this may well stand comparison with the best of such surveys, such as that of the Highwood workers, or those of Wallgren, Geddes-Dahl, Holmdahl, and others. But here again it has not been possible to take into account the profound effect of drug therapy on infection rate due to shrinkage of the reservoirs of infection, and on the prognosis as regards morbidity and mortality. However, even as they stand both these volumes should prove most useful additions to the literature on this subject.

L. J. GRANT.

Psychopharmacology: Pharmacologic Effects on Behaviour. Edited by Harry H. Pennes. (Pp. xiii + 362 ; 35 figures, 49 tables. 60s.) London: Cassell. 1958.

Within the past few years, symposiums on many subjects have been held, and the proceedings are subsequently published. One such meeting was held in the United States when the subject was psychopharmacology, in which some 41 persons took part. The 13 chapters are mainly devoted to the effects of various drugs on chemical mechanisms of the brain with special reference to mental disorders.

A few chapters on physiological findings or on clinical appraisals of the effect of the drugs are also included.

The general standard of communications is high, and it is good to see clinical psychiatrists realizing that chemical processes may be related to mental disease.

This is a volume that should be read by those whose interests lie in this field.

J. N. CUMINGS.

Books Received

Laboratory Applications in Clinical Pediatrics. By Irving J. Wolman. (Pp. xi + 1,019. £5 16s. 6d.) London : McGraw-Hill. 1957.

The Nature of Brucellosis. By Wesley W. Spink. (Pp. xiv + 464 ; 56 figures. 63s.) Minnesota University Press (London : Oxford University Press). 1957.

Microtechnique of Clinical Chemistry for the Routine Laboratory. By Samuel Natelson. (Pp. xv + 484 ; 160 figures. 82s. 6d.) Oxford : Blackwell Scientific Publications. 1957.

J. clin. Path. (1959), **12**, 101.

A CRITICAL REVIEW OF HUMAN HAEMOGLOBIN VARIANTS

PART II: INDIVIDUAL HAEMOGLOBINS

BY

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From the Medical Research Council Laboratories, Hampstead, London, N.W.3

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The authors believe that a reconsideration of some of the results of investigations so far carried out on individual haemoglobins would be salutary. A summary of previous work on their separation and characterization is therefore given, but without detailed discussion, since it is hoped that the general comments of Part I (Beaven and Gratzer, 1959) will suffice.

The possibility has been raised (Ager, Lehmann, and Vella, 1958b ; Benzer *et al.*, 1958) that samples of any given haemoglobin variant derived from different ethnic sources may be similar only in terms of particular properties, and that in reality many more haemoglobins than those enumerated below exist.

Haemoglobin A Components

Separation.—A minor component (A_2) has been separated from normal adult haemoglobin in starch block electrophoresis by Kunkel and co-workers (Kunkel and Wallenius, 1955 ; Kunkel and Bearn, 1957 ; Kunkel *et al.*, 1957), Rosa (1958), Silvestroni, Bianco, and Muzzolini (1957), and by Bernasconi and co-workers from haemoglobin of normal adults and from subjects with pathological disorders (Bernasconi, 1956 ; Marinone and Bernasconi, 1956, 1958 ; Marinone *et al.*, 1956). (Cabannes *et al.*, 1957a, have found as much as 27% in one pathological case), by Gerald and Diamond (1958a), Masri *et al.* (1958), Josephson *et al.* (1958), and Ceppellini *et al.* (1958).

A variable minor component has been found by Berry and Chanutin (1957, 1958) using free boundary electrophoresis, and fast minor components have been found by the same technique by Hoch (1950) and by Shooter and Skinner (1956). Separation and estimation of minor components by paper electrophoresis have been achieved by Aksoy, Lehmann, and Eng (1957), Silvestroni and Bianco (1957), Modiano (1957), Hoch and Barr (1956), de Traverse *et al.* (1958), and by Cabannes *et al.* (1957a). Two minor components, one fast and one slow-running, have been obtained by Morrison and Cook (1955b, 1957) by ion exchange chromatography, and by the same technique Allen *et al.* (1958) found three fast-running components but no slow ones and Huisman *et al.* (1958) one, or possibly two, fast and one slow ; three components in all were reported by Van Fossan (1954) on alumina columns (but see also Prins and Huisman, 1956a). Derrien and

Reynaud (1953) found six components by free boundary electrophoresis, but these results have been shown by Itano (1956) not to be valid (see also N.A.S.-N.R.C. Conference, 1958, p. 199). Separation of minor components in starch gel is reported by de Grouchy (1958) and Labie, Rosa, Dreyfus, and Schapira (1958). Some five components have been claimed by Derrien and co-workers from solubility measurements (Roche and Derrien, 1951, 1953; Derrien, 1957; Derrien in N.A.S.-N.R.C. Conference, 1958, p. 183), and two by Allison and Tombs (1957) (see Itano (1953a) and the discussion in Part I). Recently Masri *et al.* (1958) have obtained an "A_4" component as well as A_3 on starch, but since its proportion is so small that it can be detected only in samples of 500 to 1,000 mg. little can be said about its identity as a haemoglobin rather than a complex denaturation product (*cf.* also Ceppellini *et al.*, 1958).

Alkali-resistant components have from time to time been claimed to be present in normal adult haemoglobin (Singer *et al.*, 1951; Huisman *et al.*, 1955a; Künzer, 1955), and for the most part these have been discussed by Betke *et al.* (1954), the present authors (*v.* Part I), and others. The suggestion by Roques and de Prailauné (1953) that 8–18% of normal adult haemoglobin is alkali resistant on the basis of denaturation following treatment with formaldehyde receives no support from other directions. The stepwise nature of the denaturation reaction after treatment with formaldehyde is described by Brada (1957).

Characterization.—A preparation of crystalline haemin from Hb-A has been reported by Morrison and Cook (1955a); this is said to be heterogeneous, there being one major component (90%) and three minor ones. In addition these authors claim that one of the minor components of haemoglobin isolated from their ion exchange columns gives a different haem from that of Hb-A. Morrison and Cook deduce that heterogeneity of normal adult haemoglobin arises partially from differences of the prosthetic group. Such a conclusion is not to be taken for granted, however, since, as already pointed out, minor components could well arise through partial denaturation, oxidation or binding, and the effect of such factors on the fission of the haem-globin linkage and the nature of the haem is unpredictable. Such a conclusion finds no support in the work of Kunkel and co-workers (Kunkel *et al.*, 1957; Ceppellini, 1956), who have prepared the A_2 fraction in bulk and have carried out much careful characterization. The homogeneity of the sample is said to be uncertain, but it is found to resemble Hb-A in its sedimentation rate, visible spectrum, solubility, and crystal structure. It is also immunologically related to Hb-A. It cannot be generated from Hb-A even by quite drastic treatment. An unresolved discrepancy has been observed between the concentration of Hb-A_2 as determined spectroscopically and the concentration of total protein as estimated by a modified Folin reaction.

The minor components of Allen *et al.* (1958) have been partially characterized in terms of *iso*leucine content (*v.* Part I). The A_3 of Kunkel and co-workers is believed to be a first physiological degradation product of Hb-A (Kunkel and Bearn, 1957).

Haemoglobin F

Separation and Identification.—This has been attempted by the usual physical methods as well as by removal of Hb-A from cord blood by fractional alkali denaturation (e.g. Chernoff, 1953a).

Electrophoresis.—A description of the behaviour of Hb-F on paper has already been given. Too many unsuccessful attempts to separate haemoglobins F and A by this means have been published to mention here. Some success in the identification of Hb-F on paper has been described by Vecchio (1953), Reynaud (1953), Haynie *et al.* (1957), Gatto and La Grutta (1955a), and Pannain (1958).

Good separation has been achieved on starch gel by Owen and Got (1957), de Grouchy (1958), and de Grouchy *et al.* (1958) and on agar by Robinson *et al.* (1957), as well as by an isoelectric line spectra method by Tuttle (1956). A particularly striking separation on starch block at high voltage has been reported by Künzer and Ambs (1958). The behaviour of Hb-F in free boundary electrophoresis has been described by Hoch (1950), Zinsser (1952), Penati, Ambrosino, Liberatori, Lovisetto, and Turco (1955a), van der Schaaf and Huisman (1955b), and by others.

Chromatography.—The paper chromatography of haemoglobins has been described by a number of workers. It is doubtful whether this technique can be put to reliable diagnostic use (Vella and Lugg, 1957; Fiori, 1957), but distinction between haemoglobins A and F has been claimed by Rapi *et al.* (1956), Illari and Marenghi (1955), Penati *et al.* (1954a, 1954b), Sansone and co-workers (1950, 1951, 1952), and Benhamou and Pugliese (1958). Attempts at fractionation on starch columns have been unsuccessful (Penati

et al., 1955e). Separation by ion exchange chromatography for diagnostic (Huisman and Prins, 1955, 1957; Prins and Huisman, 1955) and preparative purposes (Huisman and Prins, 1955; Morrison and Cook, 1955b, 1957; Cook and Morrison, 1956; Allen *et al.*, 1958; Huisman *et al.*, 1958; Saha, 1959) has been described but is believed by the present authors to be often open to criticism (*v.* Part I).

Characterization.—The presence of several components in foetal haemoglobin has been suggested: (i) On the basis of irregularities in the alkali denaturation curve (White, F. D., *et al.*, 1950; Betke, 1951; Kleinknecht, 1953); the validity of this argument has been disproved by Rossi-Fanelli *et al.* (1955a), Kubowitz (1957), Betke (1952a) and others; (ii) on the basis of salting-out experiments by Roche, Derrien and co-workers (Roche and Derrien, 1951; Roche *et al.*, 1953a, 1953b, etc.); (iii) by Van Fossan (1954) on the grounds of its behaviour on alumina columns (but *v.* Prins and Huisman, 1956b); and (iv) on ion exchange resin columns by Prins and Huisman (1956) (though not on the modified cellulose columns of Huisman *et al.*, 1958) and more particularly by Allen *et al.* (1958). The latter authors report differences in the *iso*leucine contents of the fractions.

The alkali denaturation of Hb-F has been the subject of much study by Haurowitz, Hardin, and Dicks (1954), Betke (1953a), Itano (1957b), Kubowitz (1954, 1957), Golden and Layton (1956), Polosa and Motta (1955), White and Beaven (1954), Beaven *et al.* (1956), Singer *et al.* (1951), Jonxis and Huisman (1956), and others. The alkali denaturation of the carboxy form has also been studied by Derrien and Laurent (1955b), Putignano and Cognetti (1952), Beaven *et al.* (1958), Polosa, Motta, and Falsaperla (1957a), and Künzer (1953a, 1955). The last author (1953a) also studied the behaviour of cyanmethaemoglobin and methaemoglobin, and the denaturation of the reduced form has been commented on by Kubowitz (1957) and Betke (1951). The balance of evidence seems to indicate that the alkali denaturation reactions of the carboxy and met derivatives are not of practical value. The denaturation of Hb-F in erythrocytes in blood films has also been demonstrated (Kleihauer, Braun, and Betke, 1957). Acid denaturation has been studied by Putignano and co-workers (Putignano and Martino, 1951; Putignano and Cognetti, 1952), Penati *et al.* (1955c), Chini, Perosa, Putignano, and Bini (1954) and Kleihauer (1957). Foetal haemoglobin appears to be up to three times more resistant than adult, but the method is not sufficiently sensitive to be of analytical value (Penati *et al.*, 1955c).

Differences between haemoglobins F and A have also been established by Gardikas *et al.* (1953) in relation to their rates of denaturation by urea and sodium salicylate, though White and Kerr (1957) record that differences in behaviour of Hb-F and Hb-A are trivial with urea, nicotinamide, guanidine, sodium benzoate, and sodium salicylate. Heat denaturation and coagulation of the two proteins have been compared by Betke (1953c) and by Betke and Greinacher (1954b).

The solubility curves of Hb-F have been examined by Derrien and co-workers with the results already mentioned, by Giraud, Orsini, and Le Poullain (1956), Polosa *et al.* (1957c, 1957d), and Pagliardi *et al.* (1954), all using the salting-out procedure, and by Itano (1953a), White and Beaven (1954), Jope and O'Brien (1949), and Wyman, Rafferty, and Ingalls (1944). Laurent, Bouscayrol, Dunan, and Borgomano (1956) have carried out salting-out experiments using adult, foetal, and thalassaemic methaemoglobins, and observe differences in the shapes of the curves compared with the corresponding carboxyhaemoglobins.

The crystal habits of haemoglobins F and A are discussed by Jope and O'Brien (1949) and by Perutz *et al.* (1951).

The nature and significance of the oxygen dissociation curves of adult and foetal haemoglobins are discussed by Allen *et al.* (1953).

A number of specific chemical differences also exist, most of which have already been mentioned. In particular very exhaustive studies have been made of oxidation and reduction reactions in the haemoglobin-methaemoglobin system with a number of reagents and the results discussed in relation to oxygen dissociation behaviour. This work is due to Betke (1952b, 1953b, 1957). Künzer and co-workers have commented on the oxidation reaction with nitrites (Künzer *et al.*, 1953), chlorates (Künzer and Saffer, 1954), and on spontaneous oxidation of solutions of Hb-A and Hb-F (Künzer and Künzer, 1952). This reaction has also been examined by Betke *et al.* (1956d).

No differences in the affinities of Hb-A and Hb-F for isocyanides were reported by Murayama (1955).

The characteristic feature of the Hb-F ultraviolet absorption spectrum is well known: the tryptophan band anomaly and its application to analytical work is described by Beaven and

Holiday (1952), Rich (1952), and Penati *et al.* (1954d). Intensity variations in the ultra-violet absorption spectra of mixtures of Hb-A and Hb-F have also been examined by Penati *et al.* (1955d). The infra-red spectrum has been studied by Greinacher *et al.* (1953), who find no significant differences between haemoglobins F and A, and by Penati *et al.* (1955b), who report small but consistent differences.

Andersch *et al.* (1944) reported differences in sedimentation behaviour, but this has not been confirmed (Taylor and Swarm, 1949) by diffusion studies.

Betke and Greinacher (1955) have recorded differences between the adsorption capacities of alumina for haemoglobins A and F, and Jonxis (1939) found small differences in the spreading rate in monolayers. Betke (1950) has discovered a difference also between the peroxidase activities of the two haemoglobins, the magnitude of which does not seem sufficient to permit of its analytical application by the benzidine colour reaction.

The immunological specificity of Hb-F has been established (Darrow *et al.*, 1940; Vecchio and Barbagallo, 1950; Sansone and Durando, 1951; Chernoff, 1953b; Aksoy, 1955; Diacono and Castay, 1957). The number of titratable sulphydryl groups under various conditions has been reported by Murayama (1957a, 1957b, 1958) and Hommes *et al.* (1956); the latter authors have drawn conclusions as to the structure of the molecule (*v.* Part I). End group analyses have been carried out by Schapira and Dreyfus (1954), Masri and Singer (1955), Huisman and Drinkwaard (1955), Schroeder and Matsuda (1958), and Huisman and Dozy (1956). Complete amino-acid analyses are described by Rossi-Fanelli *et al.* (1954), Dustin *et al.* (1954), van der Schaaf and Huisman (1955a), Huisman *et al.* (1955b), and Stein *et al.* (1957); the globin alone has been examined by Dustin *et al.* (1954) and Perosa (1950). Dustin *et al.* (1954), Huisman *et al.* (1955b), and van der Linden (1950) discuss the *iso*leucine content, which is the greatest structural difference between Hb-F and other haemoglobins. The conclusions of Derrien and Laurent (1955a) as to the presence of Hb-F in normal adult blood appear to be doubtful (*v.* Part I). The amino-acid composition of the alkali-resistant fraction occurring in sickle-cell anaemia haemoglobin has been examined by Huisman *et al.* (1954).

There remains the perennial question whether Hb-F is identical with the alkali-resistant haemoglobin associated with thalassaemia. This has been the subject of prolonged controversy, but only a few of the most cogent recent arguments will be quoted. Briefly, the evidence for the separate identity of the alkali-resistant haemoglobin of thalassaemia is as follows: (i) Differences in free boundary electrophoresis patterns observed by Derrien and Reynaud (1955); these results, however, were obtained in cacodylate buffer under conditions already discussed, and the possible effects of differences in other cell constituents should also be considered. The Tiselius electrophoresis experiments recently described by Derrien (N.A.S.-N.R.C. Conference, 1958, p. 195) showed heterogeneity effects only after some 20 hours. As Itano (*ibid.* p. 199) points out, such a separation would correspond to minute charge differences such as could be brought about by a change in pK of one group. Moreover, in the course of excessively long runs artefacts arising from diffusion, electrolysis, and other effects are to be expected. (ii) Differences in the alkali denaturation rates of the carboxyhaemoglobins (Vecchio, 1946; Putignano and Cognetti, 1952), a technique which, as already stated, seems to be unreliable. (iii) Differences in the acid denaturation rates of the oxy and carboxy derivatives (Perosa and Bini, 1954; Chini *et al.*, 1954); here again, as Penati *et al.* (1955c) point out, the method is too insensitive for any reliable conclusions to be drawn. (iv) A difference in immunological behaviour (Diacono, 1957), and (v) alleged small differences in the rates of migration on paper (Perosa and Bini, 1954); the imponderable factors involved in paper electrophoresis again make results of this kind open to criticism. All this evidence rests on the use of ambiguous techniques. Against this may be set the many properties of thalassaemia haemoglobin identical with those of Hb-F of cord blood. These are summarized by Liquori (1951), Liquori and Bertinotti (1951), Huisman *et al.* (1956), and de Marco and Trasarti (1957), and include crystal structure, alkali denaturation rates, solubility of the reduced, oxy- and carboxy haemoglobins, spectral features, i.e., position of the tryptophan band, electrophoretic and chromatographic behaviour, and amino-acid composition. The authors believe the identity to be established beyond reasonable doubt.

Haemoglobin S

Since the identification of Hb-S as a new molecular species by Pauling, Itano, Singer, and Wells in 1949, this haemoglobin has been more thoroughly studied than any of the subsequently discovered pathological variants.

The separation of Hb-S from Hb-A by paper electrophoresis is good (Bergren *et al.*, 1954; Larson and Ranney, 1953; Reynaud, 1953; Spaet, 1953; Gatto and La Grutta, 1955b; Smith and Conley, 1953; etc.), and it is also clearly demarcated in agar (Robinson *et al.*, 1957; Giri and Pillai, 1956), starch slab (Kunkel *et al.*, 1957), starch gel (Owen and Got, 1957; de Grouchy, 1958), and by isoelectric line spectra (Tuttle, 1956). Its behaviour in free boundary electrophoresis is also distinctive (Itano and Neel, 1950; Wells and Itano, 1951; Sturgeon *et al.*, 1952; Zinsser, 1952; Singer *et al.*, 1954; Shooter and Skinner, 1955), and indeed the first fractionation was achieved by this method (Pauling *et al.*, 1949). The mobility variations observed by Anderson and Griffiths (1954), and ascribed to complex formation, have been considered in Part I. The behaviour of Hb-S on ion exchange columns appears also to be unambiguous (Morrison and Cook, 1955b, 1957; Huisman and Prins, 1955, 1957), and separation by paper chromatography has been claimed by Benhamou and Pugliese (1958).

Roche *et al.* (1952a) have attempted to show by salting-out curves that Hb-S is heterogeneous, but the criticisms already mentioned of these methods apply here. Goldberg (1956) found a double peak in the zone associated with Hb-S in paper electrophoresis, but no heterogeneity has been found by other methods. Havinga and Itano (1953) have shown that the globin of Hb-S is homogeneous.

In most respects the differences between Hb-S and Hb-A are small. Betke and Greinacher (1955) have found that the rate of oxidation with various reagents is in both cases identical, that heat denaturation and coagulation occurs somewhat faster in Hb-S, and that there are slight differences in adsorption affinity on alumina. Although the oxygen dissociation curve of blood from subjects suffering from Hb-S diseases is abnormal, this appears to be due to the presence of dialysable constituents, and the inherent oxygen affinity seems to be normal (Wyman and Allen, 1951; Becklake *et al.*, 1955). Attempts have been made to establish immunological specificity, but these have met with some difficulty (Goodman and Campbell, 1953; Diacono and Castay, 1956a, 1956b). Recently, alleged spectral anomalies have been reported (McCord and Gadsden, 1958).

Differences in the number of titratable sulphydryl groups have been found by Ingbar and Kass (1951), Murayama (1956, 1957a, 1957c) and Hommes *et al.* (1956). Terminal residues have been estimated by Huisman and Drinkwaard (1955), Masri and Singer (1955), Huisman and Dozy (1956) and Havinga (1953). Amino-acid analyses by Schroeder *et al.* (1950), Huisman *et al.* (1955b), and Stein *et al.* (1957) have shown no significant differences from Hb-A, but a difference in the composition of one peptide from partial hydrolysis was established by Ingram (Ingram, 1957, 1958; Hunt and Ingram, 1958b) by his finger-printing technique. Hunt and Ingram (1958a) find the trypsin-resistant portions of Hb-S and Hb-A to be identical. The "differential titration" method of Scheinberg *et al.* (1954, N.A.S.-N.R.C. Conference, 1958, p. 277) has been applied to Hb-S (*v.* Part I). In addition Havinga (1953) demonstrated the presence of small amounts of phosphorus in this protein. He has also noted that the fission of the haemoglobin linkage is more difficult than in Hb-A. The globins from bloods containing Hb-S have also been prepared and examined by Havinga and Itano (1953).

The most interesting and distinctive property of Hb-S, and the one from which its physiological manifestations arise, is its low solubility in the reduced state. This difference does not extend to the oxy form (Dervichian *et al.*, 1952b; Itano, 1953a), or to the methaemoglobin (Perutz and Mitchison, 1950), but in the reduced state the solubility is such that tactoids are formed in solution (Harris, 1950). The striking optical properties of sickled cells (Perutz and Mitchison, 1950) are attributed to intra-erythrocytic crystallization of the Hb-S. The behaviour of reduced Hb-S and its crystallography have been studied by Perutz *et al.* (1951), and the viscosity changes which accompany deoxygenation, and which are *p*H-dependent, are described by Greenberg, Kass, and Castle (1957) and by Griggs and Harris (1956). The effect on the solubility of other haemoglobins which may be present is important (Singer and Fisher, 1953; Singer and Singer, 1953). The viscosity of reduced haemoglobins in solution has been investigated by Allison (1957), who refers to mutual interactions and discusses structural implications. Oxyhaemoglobins A and S behave identically in respect of viscosity (Nakamura, 1955). The relatively high oxygen tension at which cells containing a mixture of Hb-S and Hb-C sickle is of interest (Allison, 1956). The same author has also made a study of the rate of sickling in relation to the proportion of Hb-S present. It is found that the latter governs the rate of sickling directly. The high osmotic and mechanical fragility of cells containing Hb-S (Lange *et al.*, 1951) arises

presumably from the crystallization of the haemoglobin in the reduced state, though it is likely that the nature of the stroma is also relevant. It may be noted that Dervichian *et al.* (1952a, 1952b) and Ponder (1951) take a rather different view of the sickling phenomenon, believing that the birefringence is the consequence of an " ultrastructure " formed by the interaction of haemoglobin with lipoproteins of the cell walls. This theory is in some measure confirmed by the electron microscopic studies of Bessis, Nomarski, Thiéry, and Breton-Gorius (1958). Perosa, Ramunni, Birri, and Manganelli (1957) report the appearance of crystals in red cells by phase contrast microscopy only when Hb-A is present as well as Hb-S.

Haemoglobin C

Hb-C separates from Hb-A in paper electrophoresis as shown by Bergren *et al.* (1954), Spaet (1953), Smith and Conley (1953), Schneider (1954), Ranney *et al.* (1953), etc. It has also been separated on agar (Robinson *et al.*, 1957), by isoelectric line spectra (Tuttle, 1956), and by free boundary electrophoresis (Ranney *et al.*, 1953; Schneider, 1954; Singer *et al.*, 1954, etc.). It moves more slowly than Hb-A on ion exchange columns (Morrison and Cook, 1955b, 1957; Huisman and Prins, 1955, 1957) and is said to separate in paper chromatography (Benhamou and Pugliese, 1958).

Ranney *et al.* (1954) found the sedimentation rate to be similar to that of Hb-A, and Betke (1957) stated that its rate of oxidation with potassium ferricyanide also shows no difference. Independent solubility measurements have been carried out but do not agree. Huisman *et al.* (1955c) report that the solubility of carboxyhaemoglobin C in phosphate buffers at *p*H 6.5 is lower than that of carboxyhaemoglobins S, A, and F.

On the other hand, Derrien *et al.* (1955a) find the solubility under very similar conditions to be high (the latter authors also find three Hb-C components). Again Itano (1953a) and Thomas, Motulsky, and Walters (1955) report the solubility of reduced Hb-C to be higher than Hb-A, whereas Huisman *et al.* (1955c) give the solubility as being slightly lower. The low solubility would be consistent with the formation of intra-erythrocytic crystals in blood containing Hb-C (Diggs, Kraus, Morrison, and Rudnicki, 1954), and the crystallization of the haemoglobin in citrated blood *in vitro* (Kraus and Diggs, 1956). Itano *et al.* (1956) suggest that these conflicting results may be explained by the existence of two different haemoglobins of identical mobility.

The number of titratable sulphydryl groups is found to be the same in Hb-C as in Hb-A (Murayama, 1957a, b, 1958; Hommes *et al.*, 1956). Terminal residues have been determined by Huisman and Drinkwaard (1955), Masri and Singer (1955), and by Huisman and Dozy (1956) with carboxypeptidase. Amino-acid analyses have been carried out by Huisman *et al.* (1955b, 1955c) and by Stein *et al.* (1957), and a difference in the composition of a peptide unit has been discovered by Hunt and Ingram (1958b). The " differential titration " method of Scheinberg *et al.* (1954) has also been applied to Hb-C, but these authors' conclusions as to the number of free carboxyl groups are inconsistent with the amino-acid results.

An increased fragility in cells containing Hb-C has been reported (Spaet *et al.*, 1953).

Haemoglobin D

Hb-D can be separated from Hb-A by paper electrophoresis as shown by Itano (1951), Bergren *et al.* (1954), Polosa and Motta (1957), Chernoff (1958), etc. Both on paper and in free boundary electrophoresis (Itano, 1951) as well as starch gel (de Grouchy, 1958) it is indistinguishable from Hb-S but is reported to run in the Hb-A position in agar electrophoresis (Robinson *et al.*, 1957). According to Huisman and Prins (1957) it is also identical with Hb-S in its behaviour on ion exchange columns. Itano (1951) and Derrien, Cabannes, Laurent, and Roche (1955b) distinguished between haemoglobins S and D by differences in solubility: whereas the solubility of the carboxyhaemoglobins is similar, in the reduced form Hb-D has a solubility which is an order of magnitude greater than that of Hb-S. Derrien *et al.* (1955b) again find at least two components to be associated with Hb-D. Immunologically Hb-D is found to be indistinguishable from Hb-A (Chernoff, 1958). As already mentioned, Benzer *et al.* (1958) found differences in the amino-acid composition of samples of Hb-D from three different sources.

Haemoglobin E

Hb-E migrates on paper at the same rate as A_2. Cabannes *et al.* (1957a) claim, however, to distinguish between them at *p*H 6.5, though such conditions are unfavourable for paper electrophoresis (Itano *et al.*, 1956). Separations of Hb-E from Hb-A on paper are given by

Chernoff *et al.* (1954), Itano *et al.* (1954), Vella (1957), and, in an extensive survey of populations in which the variant occurs, by Brumpt, de Traverse, Brumpt, and Coquelet (1957a and b) and Brumpt, Brumpt, Coquelet, and de Traverse (1958). Itano *et al.* (1954) also separated and isolated Hb-E from the ascending limb of a free boundary electrophoresis apparatus, and separation in starch gel has been described (de Grouchy, 1958). Its chromatographic behaviour is described by Prins and Huisman (1956b ; Huisman and Prins, 1957) and Morrison and Cook (1955b, 1957). The solubility of Hb-E is similar to that of Hb-A (Itano, 1953b ; Sturgeon, Itano, and Bergren, 1955).

Huisman and Drinkwaard (1955) give the number of valine residues per molecule of Hb-E as 5, and Jonxis, van der Schaaf, and Prins (1956) find that its amino-acid composition is identical with that of Hb-A. Ingram (cited in Ager, Lehmann, and Vandepitte, 1958a) has found that, although haemoglobins E and A_2 appear indistinguishable by all physical and chemical criteria so far applied, there are differences between these proteins in terms of amino-acid sequences.

Increased erythrocyte fragility has been reported to be associated with the presence of Hb-E (Chernoff *et al.*, 1954). The haemoglobin is also precipitated with brilliant cresyl blue or sodium metabisulphite (Vella, 1957), and inclusion bodies have been observed in a case of Hb-E/thalassaemia (Minnich, NaNakorn, Tuchinda, Wasi, and Moore, 1956).

Haemoglobin G

The behaviour of Hb-G in paper electrophoresis is described by Chernoff (1955), Schwartz and Spaet (1955), Schwartz, Spaet, Zuelzer, Neel, Robinson, and Kaufman (1957), Edington and Lehmann (1954), Edington *et al.* (1955), and Ager *et al.* (1958a). A comparison with Hb-D is given by Cabannes, Raffi, Boineau, Domenech, and Guivarc'h (1958). Resolution has also been claimed by free boundary electrophoresis (Edington *et al.*, 1955), and this variant is said to migrate between haemoglobins A and E on ion exchange columns (Ager and Lehmann, 1957). The solubility of reduced Hb-G is found by Edington *et al.* (1955) and Edington and Lehmann (1954) to be much higher than that of Hb-S and slightly lower than Hb-A. Crystals of Hb-G and mixed crystals of haemoglobins A and G have been prepared but not examined crystallographically (Edington *et al.*, 1955).

Haemoglobin H

This " fast " haemoglobin and its separation by paper electrophoresis has been described by Rigas *et al.* (1955, 1956). It has also been examined by free boundary electrophoresis (Rigas *et al.*, 1955, 1956 ; Thorup, Itano, Wheby, and Leavell, 1956), by starch slab electrophoresis (Hedenberg, Müller-Eberhard, Sjölin, and Wranne, 1958), and by ion exchange chromatography (Ager and Lehmann, 1958). Its isoelectric point under specified conditions is given by Rigas *et al.* (1956) as 5.6 as against 7.05 for Hb-A. It is less soluble than Hb-A in the reduced form (Rigas *et al.*, 1955 ; Vella, 1957). It is evidently unstable and is readily precipitated irreversibly from its solutions and denatured by freezing (Rigas *et al.*, 1955). The rate of this process increases with decreasing *p*H or oxygen pressure (Rigas *et al.*, 1956 ; Vella, 1957). The formation of inclusion bodies is also associated with this haemoglobin (Gouttas, Fessas, Tsevrenis, and Xefteri, 1955 ; Vella, 1957). Its presence in erythrocytes is associated with elevated fragility (Vella, 1957 ; Brain and Vella, 1958). The amino-acid composition is said by Huisman (1958) to differ appreciably from Hb-A.

Haemoglobin I

The separation of a haemoglobin designated Hb-I is described by Rucknagel, Page, and Jensen (1955) and by Cabannes *et al.* (1957b). It has been examined by free boundary and paper electrophoresis (Rucknagel *et al.*, 1955 ; Ager *et al.*, 1958a) and ion exchange chromatography (Huisman and Prins, 1957), and its sedimentation behaviour, oxygen dissociation, and solubility are reported (Rucknagel *et al.*, 1955) to resemble those of Hb-A.

Haemoglobin J

This was discovered by Thorup *et al.* (1956), and its migration on paper and in Tiselius electrophoresis is described by these authors. Patterns on paper are also shown by Ager and Lehmann (1958), and its behaviour in starch gel is described by de Grouchy (1958). Its chromatographic properties have been investigated by Huisman and Prins (1957) and Huisman, Noordhoek, and da Costa (1957). Thorup *et al.* (1956) report that its solubility in the reduced state is substantially greater than that of Hb-A. Huisman (1958) gives the amino-acid composition of Hb-J, which resembles that of Hb-A fairly closely.

Haemoglobin K

This designation was first applied to a fraction separated by Battle and Lewis (1954) by free boundary electrophoresis, no clear result being obtained on paper. Itano *et al.* (1956) were unable to obtain any such indications of a new species with the same sample, though Hb-F appeared to be present. The designation has since been applied to the so-called Liberia II haemoglobin found by Robinson *et al.* (1956), Vella (1958), and Trincão, Almeida Franco, Gomes, and Ferreira (1958), as well as apparently by Cabannes *et al.* (1957b) and Cabannes and Buhr (1955). The migration on a column is said to be slightly faster than Hb-A (Ager and Lehmann, 1958). No characterization studies appear to have been carried out. Robinson *et al.* (1956) comment that electrophoretic examination was impeded, because, when the haemoglobin solution was diluted with cacodylate buffer, a precipitate appeared.

Haemoglobin L

This was reported by Ager and Lehmann (1957) and migrates more slowly than Hb-A in paper and free boundary electrophoresis (Ager *et al.*, 1958a ; Ager and Lehmann, 1957) and on columns (Ager and Lehmann, 1957, 1958). Its solubility is said to be similar to that of Hb-A (Ager and Lehmann, 1957). The amino-acid composition as given by Huisman (1958) is somewhat similar to Hb-A.

Haemoglobin M

This was obtained as the methaemoglobin by Hörlein and Weber (1948, 1951), and was characterized by these authors, as well as by Kiese *et al.* (1956) and Heck and Wolf (1958), in terms of its spectrum and that of its derivatives, oxygen binding, oxidation-reduction kinetics, and the abnormality of its globin. Gerald *et al.* (1957) believe that they have isolated the precursor haemoglobin by electrophoresis on starch block, and Pisciotta *et al.* (1957) describe a separation on paper. Rossi-Fanelli *et al.* (1957) also describe an abnormal methaemoglobin, associated with an anomalous crystal form as well as an abnormal spectrum. A second Hb-M variant has recently been described (Gerald, 1958 ; Gerald and George, 1959).

Haemoglobin N

This is the Liberia I haemoglobin reported by Robinson *et al.* (1956). Its migration on paper (Robinson *et al.*, 1956 ; Ager and Lehmann, 1958 ; Trincão, Almeida Franco, and Nogueira, 1959) and on columns (Ager and Lehmann, 1958) has been described. There are no reports of characterization studies.

Haemoglobin O

This is the Buginese X variant reported by Eng (1957). The evidence for its existence rests solely on its electrophoretic and chromatographic behaviour (Eng, 1957 ; Eng and Sadono, 1958).

Haemoglobin P

A pre-foetal haemoglobin (Allison, 1955) has been claimed to exist by Künzer (1957b), Künzer and Drescher (1956 ; Drescher and Künzer, 1953, 1954), and Halbrecht and Klibanski (1956 ; Halbrecht *et al.*, 1958), all of whom claim to have found it in young foetuses. On the other hand Walker and Turnbull (1955) have found only Hb-F in similar foetuses, and Beaven *et al.* (1951) likewise found no evidence for any additional component in Tiselius electrophoresis under conditions giving good resolution of Hb-A and Hb-F. Halbrecht and Klibanski claim that the migration of this pigment in paper electrophoresis is significantly slower than that of haemoglobins F and A, though unresolved, and that it is alkali-resistant. Künzer (1957b) and Künzer and Drescher (1956) find that its alkali resistance is intermediate between that of haemoglobins A and F, and that the tryptophan band is in the position characteristic of Hb-F.

Haemoglobin Q

This is yet another haemoglobin which is known only in terms of its migration velocity, which is said to be identical with that of Hb-L on paper and agar but differing from it in its mobility on ion exchange columns (Vella *et al.*, 1958). Its presence has been claimed only in association with Hb-H.

Galveston Haemoglobin

The evidence for the existence of this variant (Schneider and Haggard, 1957, 1958) rests entirely on its migration velocity in paper and free boundary electrophoresis ; in the first it behaves similarly to Hb-L, and in the second it is unresolved, except on addition of further Hb-A. On ion exchange columns its migration is given as slightly slower than Hb-A, but it is not resolved and no separation occurs in electrophoresis on agar gel (Schneider and Haggard, 1958).

Bart's Haemoglobin

This was reported by Ager and Lehmann (1958) to be the fastest haemoglobin yet discovered, both on paper electrophoresis and ion exchange columns. Its alkali resistance is said to be intermediate between haemoglobins A and F and its tryptophan band is nearer the foetal than the

adult position. It was found in a child in the presence of Hb-F.

South Vietnam Haemoglobin

Recently Albahary, Dreyfus, Labie, Schapira, and Tram (1958) reported a new haemoglobin in South Vietnamese. This was said to migrate on paper at *p*H 8.6 with a velocity intermediate between Hb-F and Hb-A. No separation from Hb-A, however, was observed, but the above authors claimed that the diffuseness of the zone indicated the presence of a mixture of Hb-A and the new variant. Examination of samples containing the alleged variant by starch gel electrophoresis led de Grouchy *et al.* (1958) to announce the existence of no less than three South Vietnam haemoglobins, two slightly slower and one slightly faster than Hb-A and none of them resolved.

Hopkins I and Hopkins II

Smith and Torbert (1958) have reported two electrophoretically new haemoglobins. No physical or chemical characterization is given.

Norfolk Haemoglobin

Ager *et al.* (1958b) have described a variant occurring in an English family, with an electrophoretic mobility very slightly different from Hb-A but separating from it on ion exchange columns.

Lepore Haemoglobin

This has been reported by Gerald and Diamond (1958b) as separating in the same position as Hb-S or Hb-D on starch block. The samples show no trace of abnormal haemoglobin, however, when subjected to free boundary and agar gel electrophoresis or ion exchange chromatography. Solubility measurements on the recovered pigment have been carried out.

Other Abnormal Haemoglobins

An abnormal fast haemoglobin was discovered in a child by Fessas and Papaspyrou (1957). It was not alkali-resistant, and its possible identity as Hb-I, J, or K was ruled out by genetic considerations (*v. supra*). This haemoglobin disappeared almost completely some three months after birth (*cf.* Hb-F).

A haemoglobin said to migrate on paper in a similar position to haemoglobins E and C and associated with thalassaemia in a Frenchman has also recently been mentioned (André, Bessis, Dreyfus, Jacob, and Malassenet, 1958).

Chernoff (N.A.S.-N.R.C. Conference, 1958, p. 179) also mentions a "Durham I" haemoglobin, but gives no description.

Conclusions and Practical Implications

It will be clear that the recognition of a new haemoglobin variant—and indeed in many cases the identification of an abnormal pigment with one of the hitherto accepted variants—is a matter demanding considerable caution. Workers in the field have of late recognized this fact and have exercised more restraint than previously.

Apart from the difficulties and consequent ambiguities arising out of the inherent chemistry of the haemoglobin molecule, a situation has now been reached where all the known haemoglobin variants (and nearly 30 have been named) lie within a quite narrow range of electrophoretic (and chromatographic) mobility. In the present state of protein chemistry and the almost complete absence of specific differences in chemical reactivity (apart from alkali denaturation rate) between the haemoglobin variants, electrophoresis and/or chromatography must remain the basic methods for distinguishing between such closely related molecular species. In addition, it is necessary to appreciate that these techniques have limitations, both of resolving power and reproducibility. This is particularly true of paper electrophoresis, which, having a number of obvious virtues for routine purposes, remains the most widely applied technique. It will be seen from the foregoing summary of the individual haemoglobin variants that the evidence for the existence of a number of these pigments is based largely on paper electrophoresis, in which they are said to migrate "between haemoglobins A and F." It is evident that here the limitations of the technique are being disregarded.

The same strictures apply in greater or lesser degree to the other methods available, but it seems likely that electrophoresis in agar gel and possibly in starch gel (bearing in mind the striking results which this technique has yielded for serum proteins) may with careful development give substantially greater resolution of haemoglobins. It is already clear that agar is particularly valuable in situations involving Hb-F, especially in the absence of specialized spectrographic facilities; this method should not be neglected.

Chromatography on ion exchange columns is clearly subject to the same limitations as zone electrophoresis, possibly in greater degree, since the migration rates cannot be related, even as a first approximation, to charge differences. It would appear that the fundamental principles

controlling such separations of large molecules have not yet been fully elucidated.

The recognition of a given haemoglobin variant in zone electrophoresis is rendered much more certain if reference samples are available for simultaneous comparison. This is not difficult to arrange with the commoner variants, but may not be feasible with rare or less well-established variants that have been reported on only a few occasions. The potential value of a readily accessible reference collection of haemoglobin variants is obvious, but the operation of such a scheme would encounter serious problems in the collection, identification, storage, and distribution of large numbers of samples of valuable and unstable material.

The only technique which can, in principle, characterize a haemoglobin variant in reproducible, quantitative terms is Tiselius free-boundary electrophoresis, from which a curve relating absolute mobility to *p*H under given conditions can be derived, even in the absence of reference samples. From the published data it is not yet certain whether, in fact, such measurements could be made with sufficient precision for this purpose. In view of possible complications arising from interactions in solution between haemoglobin species, it may be necessary to envisage the prior fractionation of mixtures, so that only single-component systems are characterized in this way. It is clear that such studies are not feasible for the routine examination of large numbers of samples.

As already suggested (Annotation, *British Medical Journal*, 1958, vol. 1, p. 1469) the final recognition of a haemoglobin variant as a unique molecular species may have to await the perfection of methods for the complete amino-acid analysis and determination of the residue sequence and interchain linkages. The work of Ingram and his colleagues on the separation and identification of peptides derived from haemoglobins by enzymatic hydrolysis is a notable advance along these lines.

Addendum

In a subject expanding as rapidly as the one under review, the rate at which new material accumulates defeats any attempts at completeness from the outset. The recent publications mentioned below either bear on the arguments outlined in Part I (Beaven and Gratzer, 1959) or indicate the trends of current work on the haemoglobin variants.

Much recent work has been concerned with the refinements of protein fractionation techniques as applied to haemoglobin. These include starch gel electrophoresis, which has been applied by Labie *et al.* (1958) to the evaluation of Hb-A_2, and the examination of a number of abnormal haemoglobins in a "discontinuous" system, in which one buffer is used in the preparation of the gel and another for the electrophoresis (de Grouchy, 1958); improved separation by this means has been claimed.

The use of agar at high *p*H (in the region of 8.6) has been developed and applied successfully, mainly to animal haemoglobins (Fine, Uriel, and Faure, 1956; Monnier and Fischer, 1958). Whether these procedures have advantages over the low-*p*H method of Robinson *et al.* (1957) is questionable; certainly no comparable resolution of Hb-F is observed.

Good results in the separation of haemoglobins A, S, C, and F have been claimed by an improved paper chromatography technique (Benhamou and Pugliese, 1958).

The salting-out curves of normal adult haemoglobin have been re-examined by Polosa *et al.* (1958a) and an analysis of accuracy and reproducibility is given.

A discussion of the globin structure in relation to the reversible dissociation of haemoglobins A, S, and C (see Part I, p. 13) based on ultracentrifuge and electrophoretic studies is given by Itano and Singer (1958). The most important result of this detailed work is confirmation that hybrid molecules, arising from dissociation and recombination of two different molecular species, are not formed.

A significant contribution to the investigation of the cysteine content of normal adult haemoglobin in the native and partially denatured states has been made by Cole, Stein, and Moore (1958). The results enumerated in Part I (p. 12), notably those of Allison and Cecil (1958), may be re-examined in the light of this work.

A good deal of attention has been focused of late on the non-haem proteins which have at various times been demonstrated in haemolysates (see Part I, p. 15 seq.). Two such proteins were observed by Polosa, Motta, and Pennisi (1958c), and the same authors (1958d) isolated the more abundant of them and examined its absorption spectrum, which was found to show only a protein band. This protein is presumably to be identified with the "X" component reported by Derrien *et al.* (1956), which is absent in the foetus and appears 11 months after birth. Laurent, Depieds, and Derrien (1958) present evidence, based on alkali denaturation and immunological properties

of the "X" protein, in comparison with those of serum proteins of comparable mobility, that this substance is stromal or intra-erythrocytic in origin.* The isolation of the protein by electrophoresis, and the presence of up to three other non-haem proteins, is described by Derrien, Laurent, and Reynaud (1958). The most interesting characteristic of this protein (Laurent, Borgomano, and Derrien, 1958) is its high *iso*leucine content—stated by these authors to be some six times greater than that of Hb-A and twice that of Hb-F. (But note that Stein *et al.* (1957) give the *iso*leucine content of electrophoretically purified Hb-A as less than 0.02 g./100 g. compared with about 1.45 for Hb-F, a ratio of greater than 1/70.) The protein moreover remains even after recrystallization of the haemoglobin. These observations may render suspect the characterization of fractionated haemoglobin zones in terms of *iso*leucine content (Allen *et al.*, 1958) and bear also on the discussion of effects which could arise from the binding of haemoglobin with extraneous material (Part I). One other recent publication should be mentioned in the same connexion. Anderson and Turner (1959) find that 3% of the total haemoglobin content of the erythrocyte remains integrally bound with the stroma, being inseparable by repeated washing; or, conversely, that over 50% of the total stromal material is haemoglobin. This is judged to be in the native state because drastic treatment is avoided in the preparation of the red cell "ghosts," and also because of the colour changes which accompany removal and readmission of oxygen. It is to be supposed that any stromal material which is not precipitated and remains in solution also contains bound haemoglobin, and might be expected to have a characteristic behaviour of its own in fractionation experiments.

Two reports have appeared of a new foetal haemoglobin variant. Fessas, Mastrokalos, and Fostiropoulos (1959) describe an "Alexandria" haemoglobin, present in an infant to the extent of 18.3% at birth, falling to 2.2% after 15 weeks. This pigment is said to migrate more slowly than Hb-S at *p*H 8.6 in paper and starch block electrophoresis, and similarly to Hb-S at *p*H 6.7. It is not observed in agar at *p*H 6.5 and resembles Hb-F in respect of alkali denaturation and position of the tryptophan fine-structure band. Vella, Ager, and Lehmann (1959) claimed to have observed a similar substance in a cord blood specimen of Chinese origin. Migration at *p*H 8.6 on paper was similar to Hb-S, and ion exchange chromatography and agar gel electrophoresis revealed only the zones associated with Hb-F and Hb-A. No difference from Hb-F was observed in alkali denaturation or position of the tryptophan band, but the spectra shown are not adequate to support this conclusion. Another haemoglobin variant has been claimed to be present in an infant by Bernard, Schapira, Dreyfus, Mathé, Rosa, and Labie et Cohen (1958). This is said to have mobility intermediate between haemoglobins A and F in paper at *p*H 8.6 and to run slightly more slowly than Hb-A on starch slab and slightly faster on an ion exchange column.

Further evidence has been claimed for the existence of an embryonic haemoglobin (Halbrecht, Klibanski, and Bar Ilan, 1959). It is unresolved on paper, but is said to show up on ion exchange columns, though it is not clearly visible in the published figure (no reason is given why it should not be identified with the small component frequently observed by Prins and Huisman (1956b) in cord blood under the same conditions). It is also reported to be present in cases of full-term infants with certain congenital disorders, which are supposed to be associated with the delayed disappearance of this pigment.

*It may be noted, however, that Fine *et al.* (1956) regard small components detected in agar electrophoresis as serum constituents which remain in spite of repeated washing of the erythrocytes.

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THE EFFECT OF SWEATING ON THE PROTHROMBIN TIMES OF BLOOD

BY

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Prothrombin times have been estimated in man before and after sweating, showing that prothrombin times are increased after exposure to high environmental temperatures over a period of 40 minutes.

Methods

The process used to induce sweating is described by Lyburn (1956). Two millilitres of blood were taken from antecubital veins before and after sweating and put into bottles containing 0.2 ml. of 1.34% sodium oxalate and sent to a laboratory within an hour for estimation of the prothrombin times. The method used was a modification of Quick's method, using thrombokinase "geigy" and 0.18% calcium chloride. The normal by this method is 15 to 16 seconds.

Subjects on anticoagulant therapy were given "marcoumar," 3 mg. b.d. for two days, and thereafter 1.5 mg. for two or three days until the prothrombin time reached 30 or 40 seconds. Thereafter they were maintained on 1.5 mg. five times a week.

Results

Comparison of the prothrombin times before and after sweating shows a significant rise over a period of 40 minutes. In Table I the increase in prothrombin times varied from 22 to 281 seconds, where treatment with anticoagulants and sweating was combined. The average increase in prothrombin times in these subjects was 93 seconds, but none of the subjects showed any evidence of bleeding. Table II shows five subjects in which the plasma failed to coagulate and three subjects in which the prothrombin times increased an average of 10 seconds.

Discussion

The increase of prothrombin times after sweating is significant, and Tawfic (1955) observed that anticoagulant therapy has to be more carefully watched in tropical climates. Doan (1953) observed a rise in prothrombin times in a subject who died following hyperpyrexial treatment. The efficiency of the factor V-factor VII-prothrombin system is affected by anticoagulants, and as shown in Table I this prothrombin system may also be affected by sweating and high environmental temperatures. It has been reported that prothrombin times vary over a period of days after anticoagulant therapy (Matthews, Meynell, Parsons, Warburton, and Waterhouse, 1955), but there has been no

TABLE I

PROTHROMBIN TIMES AFTER COMBINED ANTICOAGULANT AND WET THERMAL TREATMENT

Date	Age	Sex and Diagnosis	Prothrombin Time before Treatment (sec.)	Prothrombin Time after Treatment (sec.)	Average Normal (sec.)
4/8/54	70	F. Coronary thrombosis	36	59	17
7/1/55	66	M. Retinal embolism	27	58	17
17/5/54	56	F. Muscular degeneration	67	349	17
6/5/54	56	F. ,,	40	80	17

TABLE II

PROTHROMBIN TIMES AFTER SWEATING

Date	Age	Sex and Diagnosis	Prothrombin Time before Treatment (sec.)	Prothrombin Time after Treatment (sec.)	Average Normal (sec.)
20/5/55	56	M. Coronary thrombosis	17	Failed to coagulate	17
20/12/54	70	M. Angina ..	16	,, ,,	17
22/12/54	52	M. Parkinson's disease	13	,, ,,	17
20/11/54	72	M. Cardiac irregularity	16	,, ,,	17
6/5/56	73	F. Auricular fibrillation	17	,, ,,	17
20/12/54	70	F. Angina ..	13	23	17
5/5/55	56	M. Cerebral thrombosis	13	24	17
20/12/54	70	F. Auricular fibrillation	13	23	17

report of prothrombin times varying in man when exposed to constant or varying environmental temperatures.

Summary

Prothrombin times have been recorded before and after sweating with and without the use of anticoagulants in a number of subjects suffering from thrombotic manifestations. The results have shown a significant increase in prothrombin times following sweating with and without anticoagulants.

The average rise in prothrombin times following treatment combined with anticoagulant therapy was 93 seconds and without anticoagulants the average rise was 10 seconds.

In five subjects (treated without anticoagulants) blood failed to coagulate after sweating.

Bleeding did not occur in any of these subjects.

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AN ASSAY FOR THE ANTITHROMBOTIC ACTIVITY OF ANTICOAGULANTS

BY

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Anticoagulants have been used for 20 years for the treatment of thrombosis. This practice has been based on the original experiments of Pupini (1932), Murray, Jaques, Perrett, and Best (1937), and Dale and Jaques (1942) which showed that anticoagulants could prevent the formation of experimental thrombi in dogs. There has been, however, no method reported by which it is possible to produce thrombosis in small animals for rapid assessment of the incidence of the lesion with different treatments. Such a method would be of value both to assess the relative activity of different anticoagulants in preventing thrombosis and also to make possible the quantitative experimental study of factors affecting thrombus formation *in vivo*. The present study was undertaken with this purpose and describes a simple method which appears to attain these objectives.

Method

Albino rats of both sexes, weighing between 150 and 300 g., were employed. The animals were anaesthetized with ether and immobilized on their backs. The neck was clipped, and a skin incision made from the middle of the right clavicle for 1 cm. anteriorly. The subcutaneous tissues were freed with forceps to expose a section of the jugular vein 1 cm. long. Haemorrhage was controlled by light pressure with moistened absorbent cotton. Four to six drops (0.2 ml.) of 10% v/v formalin in 60% methyl alcohol were now applied to the adventitia of the vessel by means of a dropper, and the skin incision closed by clips or suture. After 24 hours the vessel was examined to determine vascular occlusion. In developing the method, a number of experiments were done in which the vessel was left 48 hours before inspection.

After the incision was reopened, the vessel was inspected carefully and the presence of a clot recorded. Finally the vessel was opened with scissors and the clot checked visually. In the case of thrombosis the vessel is dark and discoloured. Absence of flow on cutting was not used as a final criterion, since this could be due to constriction and flow sometimes persisted in the presence of a large clot incompletely obstructing the lumen.

"Depo" heparin was kindly supplied by the Upjohn Co., Kalamazoo, Michigan, in 1 ml. vials containing 20 mg. of heparin sodium, 180 mg. of gelatin, and 80 mg. of anhydrous dextrose preserved with sodium ethyl mercurithiosalicylate 1:10,000. We are indebted to Hoffman-LeRoche Inc., Nutley, New Jersey, for "treburon." This was received as a sterile solution in 2 ml. ampoules. It was an experimental preparation and 1 ml. contained 250 mg. of RO 2-3053. These ampoules were not preserved with phenol. Both of these drugs were injected subcutaneously five hours after the operation. The injection was repeated every eight hours. Doses are reported in mg./100 g. body weight given as a single injection.

"Dicoumarol" was kindly supplied by Chas. E. Frosst and Co., Montreal. This was incorporated in fox chow and fed at a level of 10 to 100 mg./kg. per day for three days. "Warfarin," supplied by S. B. Penick & Co., New York, was similarly dispensed for doses of 0.03 to 2 mg./kg. The animal cages were inspected daily to ensure that all of the food was eaten. Blood was taken for prothrombin times from the tail vein, and the prothrombin times were determined by the method of Schwager and Jaques (1949), adding 2 drops of blood to 2 drops of rabbit brain thromboplastin suspension.

Results

Apitz (1938) observed that the application of alcohol to the outer wall of the jugular vein of rabbits produced a thrombus. In preliminary experiments absolute alcohol and 10% formalin were applied to the adventitia of the jugular veins of rats. Both produced an occlusion in most of the vessels treated. The vessels found occluded at 24 hours showed a typical platelet thrombus on histological study. The method was standardized using 10% formalin in 60% methyl alcohol. The incidence of thrombosis is shown in Table I. Results are shown for three separate investigators

TABLE I

INCIDENCE OF THROMBOSIS IN NORMAL RATS PRODUCED WITH 10% FORMALIN SOLUTION

Series	Operator	No. of Rats	No. with Thrombus	% Incidence of Thrombosis
1*	OB	20	16	80
2	OB	30	22	73
3	OB	10	6	60
4	OB	10	7	70
5	JA	18	9	50
6	JA	9	7	78
7	JA	20	12	60
8	JA	19	10	59
9	JA	21	11	53
10	ER	10	7	70
11	ER	9	7	78
Total		176	114	
			Overall incidence=	64·8%

Mean incidence for 10 experiments examined at 24 hours=65·1% S.D. ±9·7.

* Veins examined 24 hours after operation except in series 1, in which rats were examined after 48 hours.

and the experiments were spread over a period of five years. The overall incidence was 64.8%. There was a slightly higher incidence when the inspection was made 48 hours after the application of the agent. There appeared to be no significant difference in the incidence of thrombosis in rats between sexes, sources of rats, or operators.

Simply stripping the vein without the addition of formaldehyde has resulted in a high incidence of thrombosis, but this varied with the operator. More consistent results were obtained with formaldehyde solution. Moreover, in a series not treated with formaldehyde, there was a very low incidence of thrombosis 48 hours later. This suggested that the thrombus had been dislodged. The evident inflammatory reaction and even necrosis observed microscopically in the formaldehyde-treated series served to fix the thrombus *in situ* and prevent this. Observations at a quarter, half, one, and six hours indicated that the occlusion of the vessel was due to the slow formation of a platelet thrombus and not to the immediate formation of an intravascular clot. In several series, portions of vein were removed, and, after routine fixation and section, examined microscopically. As indicated above, blood might flow in the presence of a thrombus, and due to vasoconstriction fail to flow in its absence, but visual inspection of the clot microscopically checked with the microscopic results. In all cases, the occlusion proved to be in part or whole a true platelet thrombus. Macroscopically, it contained a distinct pink body quite easily distinguished from the dark, almost black, intravascular blood clot. Microscopically, with haematoxylin and eosin, the occluding mass appeared pinkish with the typical pattern of a platelet thrombus and showing occasional darker masses of red cells.

Comparison of Depo Heparin and Treburon in Rats.—Groups of rats were subjected to the operation and then injected with "depo heparin" or "treburon" subcutaneously five hours later. The injections were repeated every eight hours. The results are shown in Table II. The results indicate that as the dose of "depo heparin" or of "treburon" increases, the incidence of thrombosis decreases. There were many deaths with the larger doses of anticoagulants, particularly if the rats were not examined until 48 hours after the operation. This brought about a drastic reduction in the number of animals available for examination at the higher dose levels. The haemorrhage was generally at the site of operation, but some animals showed haemorrhage at the site of injection. The relative effectiveness of "depo heparin" and "treburon" was about the same. Both drugs reduced the incidence of thrombosis to 40%. The incidence of thrombosis was converted to probits and the relative antithrombotic activity from the regression line was 0.81 : 1.00 for "treburon" : "depo heparin."

TABLE II

INCIDENCE OF THROMBOSIS AFTER SUBCUTANEOUS ADMINISTRATION OF DEPO-HEPARIN AND TREBURON FIVE HOURS AFTER OPERATION

Dose (mg./100 g.)	Depo-Heparin		Treburon	
	No. of Rats	Incidence of Thrombosis (%)	No. of Rats	Incidence of Thrombosis (%)
1	10	80	10	90
2	8	87	8	75
3	10	60	16	47
4	—	—	26	60
5	9	67	10	80
10	13	38	24	54
20	12	42	18	44
40	10	40	—	—

Veins examined after 24 hours except with 1, 2, 3 mg./100 g. dose (examined after 48 hours).

Incidence of Experimental Thrombosis after Dicoumarol and Warfarin.—Male rats were given indirect anticoagulants in varying amounts by incorporating the compounds in their diet for three days. On the third day the rats were anaesthetized lightly with ether, a tail vein puncture was made to obtain blood for prothrombin times, and the right jugular vein was exposed and treated with the formalin solution. Twenty-four hours later the rats were again anaesthetized with ether and the jugular vein was examined for thrombosis. The prothrombin

time was determined from blood taken from one of the jugular veins. The incidence of thrombosis was determined by careful examination of the vein, judging if it contained a clot both by appearance and by estimation of internal patency and blood flow.

The post-operative prothrombin times were often found to vary considerably above and below those of the first day, and since this change could not be foretold or controlled the two times were averaged. The normal prothrombin time by the method used is about 26 sec. The results are shown in Fig. 1, as is the incidence for various prothrombin times. As observed in this laboratory in other work, when dicoumarol is given to rats, there is a wide distribution of resulting prothrombin times at most dose levels. Warfarin is much more effective than dicoumarol in the rat, requiring only 1/300 by weight. Further, the response appears to be somewhat more uniform, although even with the highest dosage used one rat out of 10 gave a normal prothrombin time. This appears to be the problem of individual resistance to prothrombopenic drugs, described by Link (1943–44) with dicoumarol in rabbits.

When the percentage incidence of thrombosis was plotted against the dose of anticoagulant (Fig. 2a) there was a marked reduction in the incidence with "warfarin" and less reduction with dicoumarol. There appeared to be little correlation between dose and incidence of thrombosis. As there is poor correlation between the dose of dicoumarol and resulting mean prothrombin time (Fig. 1), the data were replotted. The rats were placed in groups according to the individual mean prothrombin time, irrespective of the amount of anticoagulant received. Any groups with fewer than 10 rats were not used. The incidence of thrombosis for these groups is shown in Fig. 2b. It can be seen that the incidence of thrombosis decreases stepwise with the increase in prothrombin time. Separate calculations for "warfarin" and dicoumarol indicate a similar relationship between their resulting prothrombin time and the incidence of

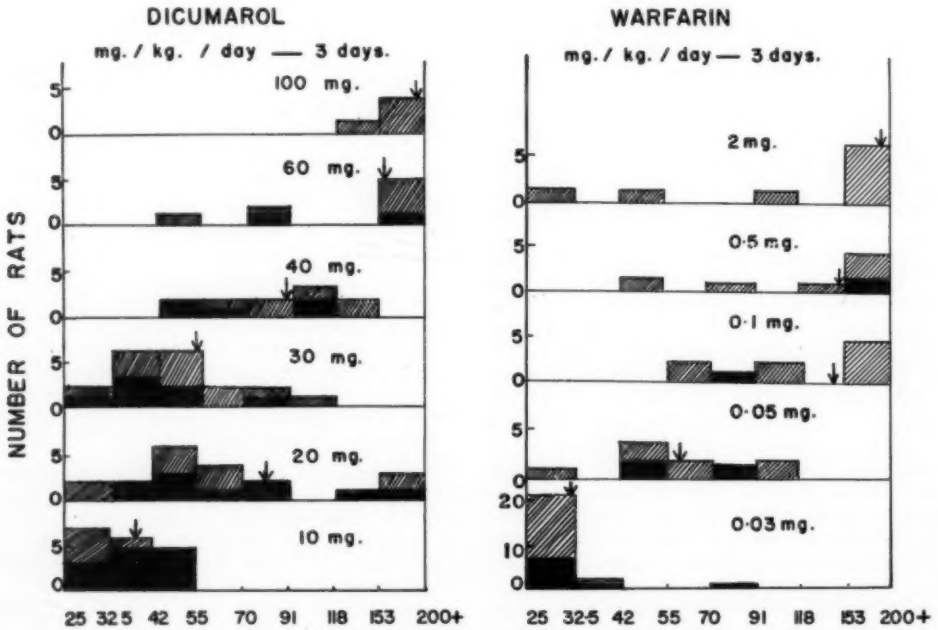

FIG. 1.—Effect of dicoumarol and "warfarin" on the prothrombin times of operated rats. Prothrombin time intervals, $\Delta T = T_0 - T_1 = 0.3 \times T_1$; with $T_0 = 25$ sec. ▩ = Number of cases with prothrombin times in range shown. ■ = Number of cases with thromboses vein. ↓ = Mean prothrombin time for any given dose of anticoagulant.

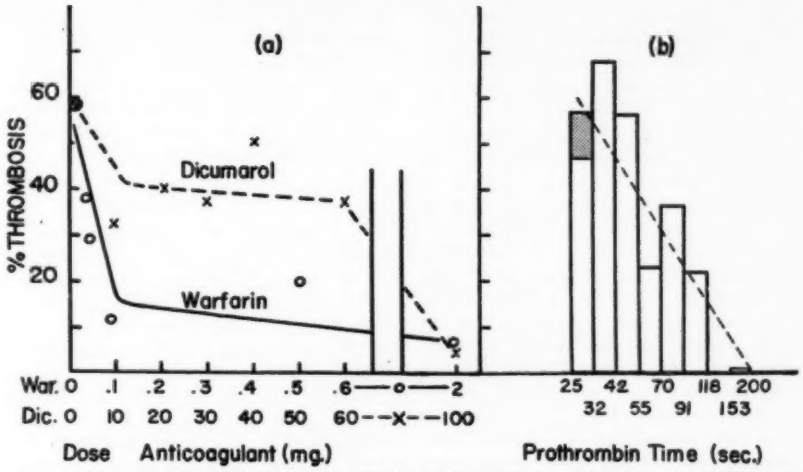

FIG. 2.—(a) Incidence of thrombosis in rats related to the dose of indirect anticoagulants. (b) Incidence of thrombosis in rats related to the prothrombin time following indirect anticoagulants. ▩ = Incidence without anticoagulant treatment.

thrombosis, but the numbers participating in some groups were not sufficient for analysis.

Discussion

Many methods have been used to produce thrombosis and intravascular clotting or intravascular clotting alone in larger experimental animals. Starting with Wooldridge in 1886, many have injected thromboplastin (tissue extracts) into dogs for this purpose (Wooldridge, 1893). Tubes of glass and collodion have been placed in the circulation, sclerosing agents, catgut, and clots have been introduced, trauma of the vessel wall, tying of the vessel, occlusion by pressure, and the application of an electrical charge have all been used. The present method appears to be the first using the rat. It has the advantage that it can be quickly and easily performed so that experiments may be designed to test the effect of various factors on the incidence of thrombosis with a measure of statistical significance. The incidence of thrombosis produced by this technique was not affected by the age or sex of the rats. It is believed that the principle used, local damage, is similar to that which frequently results in clinical thrombosis. The inflammation and necrosis of the vessel wall due to the formalin are probably contributory factors to the fixation of the thrombus. McGovern (1955) has shown that a probably adequate stimulus for thrombosis is the tissue anoxia which arises from a mere "stripping" of the vessels concerned, but we found a variable incidence when stripping alone was employed.

A thrombus can be detected in a few rats half an hour after operation, and the incidence increases thereafter. The failure of depot heparin and "treburon" to prevent completely the thrombus formation is probably due to the anticoagulants being given five hours after operation. Lately it has been shown that when heparin is given intravenously immediately after the operation, extremely small doses will completely prevent thrombosis without haemorrhage. In the series with depot heparin and "treburon," it appeared that "depot heparin" had a greater haemorrhagic activity than "treburon" (1.0:0.6) in line with its greater anticoagulant activity. Since most of the animals showed a large haematoma in the neck, it was assumed that the operation was responsible, but in a control series with anticoagulant alone it was found that the incidence of haemorrhage was just as high. The high incidence of haemorrhage in rats receiving 5 mg./100 g. "depo-heparin" was not observed by Haist (1938) with plain heparin and is in marked contrast to the general observation with other species of experimental animals, in which the blood can be rendered incoagulable with heparin without any signs of haemorrhage.

The variation in prothrombin time following administration of equal amounts of dicoumarol or of "warfarin" in the rat's food was not due to

variations in absorption. As intraperitoneal injection of "warfarin" resulted in variations in the prothrombin time similar to that following oral administration, the differences in utilization of the drug are chiefly responsible.

The fact that a relationship has been established between the prothrombin time value and the incidence of thrombosis is a significant observation. Since the initial paper of Murray *et al.* (1937) on the application of anticoagulants to the prevention of thrombosis, it has been universally assumed that there is some correlation between the degree of impaired coagulation and the reduced incidence of thrombosis following the use of anticoagulants. It has been, however, extremely difficult to obtain any evidence for this, either in experimental animals or in man, and the data presented for dicoumarol and "warfarin" are most important, showing, in rats, that, while there is no correlation between the drug dosage and reduction in incidence of thrombosis, there is a correlation between the prothrombin time produced by indirect anticoagulants and the reduced incidence of thrombosis.

What is the therapeutic level for the anticoagulants? At present there is no evidence that there is a therapeutic level at which intravascular clotting can be absolutely prevented. Several investigators reported a lowering of the incidence of experimentally produced thrombosis in anticoagulant-treated animals compared with a control series. The results obtained demonstrate that the degree of reduction in incidence of thrombosis is dependent on the degree of reduction in blood coagulability for the indirect anticoagulants. The latter is affected by the nutritional status, genetic constitution, and pre-existing hypercoagulability or hypocoagulability and hence these factors determine the dosage of drug required. Just as there is a range of prothrombin times in which the incidence of thrombosis is reduced proportional to the prothrombin time, so presumably there are varying degrees of thrombotic tendency which will require a greater degree of hypocoagulability to prevent thrombosis. If one could estimate quantitatively the degree of thrombotic tendency in different clinical conditions (post-operative, etc.), and relate this to the animal, then obviously it should be possible to predict the degree of protection required for these. However, the method described should in any case be valuable in comparing the relative antithrombotic activity of different anticoagulants.

Summary

A method is described for producing thrombosis in rats by the application of formalin to the jugular vein and inspecting the vein after 24 hours. The percentage incidence of thrombosis was 65.1 ± 9.7 for 200 g. rats.

The administration of "depo heparin" and "treburon" subcutaneously reduced this incidence of thrombosis to 40%. The ratio of antithrombotic activity thus determined for "treburon": "depo-heparin" was 0.8 : 1.0.

In the rats used, the prothrombopenic activity of "warfarin" given orally to rats over three days was about 300 times that of dicoumarol on a weight basis, as judged by the increased prothrombin time. Dicoumarol and "warfarin" reduced the incidence of thrombosis. There was little correlation between the dose of drug and incidence of thrombosis, but there was a significant correlation between the incidence of thrombosis in rats and the prothrombin time levels produced by these drugs.

We wish to acknowledge the valuable assistance of Dr. E. F. Routley, who participated in a part of the programme, and the kind encouragement and advice of Professor D. F. Moore and Dr. J. E. Newell, of the Department of Pathology, University of Saskatchewan. This work was supported with a grant from the Defence Research Board of Canada.

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THE EXTRACTION OF EVANS BLUE (T1824) FROM PLASMA AND THE MEASUREMENT OF PLASMA VOLUME

BY

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Haemolysis and lipaemia interfere with the direct photometric estimation of Evans blue in plasma. During an investigation of blood volume changes in surgical shock using this dye, lipaemia was often encountered and it was necessary to use an extraction procedure for the estimation of the dye concentration. A rapid method was desired.

The method of Crooke and Morris (1942) using a hydrochloric acid - ethanol - phosphotungstic acid reagent is quicker to use than the chromatographic procedures described by Allen (1951) and by Bedwell, Patterson, and Swale (1955). Morris (1944b), however, described certain disadvantages, including that due to haemolysis, although Morris (1944a) had described an optical correction for the presence of haemoglobin in dyed plasma using filters with peak transmission at 600 mμ and 430 mμ.

Because the original authors gave only a brief account of their experiments, the method was investigated in detail. The procedure for shaking and centrifuging was modified. Spectrophotometry at an optical depth of 4 cm. was used and a spectrophotometric correction for the presence of haemoglobin was introduced. On the basis of these experiments, which are detailed later, the following method was adopted, and was found to be free from the main disadvantages described by Morris (1944b).

Method for Measurement of Plasma Volume

Extraction Reagent.—Phosphotungstic acid (Hopkin and Williams, Analar, granular form), 7.5 g., is dissolved in 50 ml. of absolute alcohol. Then 8 ml. of concentrated hydrochloric acid (A.R.) is added slowly with shaking. The mixture is filtered through a fluted Whatman No. 1 filter paper. This reagent is best made up shortly before use, as a blue colour has been said to develop on exposure to light (Crooke and Morris, 1942).

Different preparations of phosphotungstic acid vary in their solubility in alcohol. The granular form dissolves rapidly and the resulting solution has only a small light absorption at 620 mμ. This was not the case using some other specimens of phosphotungstic acid which were coloured.

Procedure.—A sample of blood is withdrawn from a vein with minimal stasis. A solution of Evans blue (usually 20 mg. in 5 ml. for adults) is then injected through the same needle from a calibrated syringe. Ten minutes later a second sample of blood is withdrawn from a different vein. Dried heparin is a suitable anticoagulant. The plasma is separated and the concentration of dye in the second sample is determined by comparison with standards after extraction.

An initial dilution of the same sample of dye injected is made in distilled water for preparing standards. Two standards are made by adding 0.1 and 0.2 ml. of the solution to 1 ml. of the patient's undyed plasma. The resulting dye concentrations should straddle that in the second sample obtained from the patient. Into two other centrifuge tubes are delivered 1 ml. of the patient's undyed and dyed plasma respectively. Then 7 ml. of the extraction reagent is added to each tube, which is stoppered with a rubber bung and shaken vigorously by hand for 10 seconds, immediately after the addition of the extraction reagent. This breaks up the protein precipitate, which otherwise sticks to the side of the tube resulting in incomplete extraction of the dye. All tubes are then shaken horizontally in the direction of the long axis in a Kahn shaker for 10 minutes. They are then capped and centrifuged for 10 minutes at 2,500 r.p.m. The temperature in the centrifuge bucket should not rise more than 5° C., otherwise turbidities develop (see below). The supernatant fluid is removed and the test and standards are read against the blank in a "unicam" spectrophotometer SP 500 or SP 600 at 620 mμ and 400 mμ. The readings are made at an optical depth of 4 cm. using a modified cuvette holder to allow the use of a volume of 6 ml. At 620 mμ light absorption by Evans blue is maximal, and at this wavelength the dye obeys Beer's law in the extraction reagent over a range of concentrations of 0.1 to 0.4 mg./100 ml. This is a wider range than is encountered in plasma volume estimations after

the injection of 10 to 20 mg. of dye in adults. The optical density of extracted undyed plasma is very low at this wavelength. In order to correct for haemolysis in the second dyed sample, the optical density at 400 mμ is divided by an experimentally determined factor of 35 (see below). The value so obtained is subtracted from the optical density at 620 mμ. If the test solution at 400 mμ yields a negative reading, this is due to greater haemolysis in the blank undyed plasma. In this circumstance the optical density of the blank solution is read against the test solution at 400 mμ. This optical density is divided by 35 and added to that of the test solution at 620 mμ. This corrects for haemolysis in the undyed plasma.

Calculation.—Let T be the optical density of the test solution corrected for haemolysis. If V be the volume of dye injected, and S_1 and S_2 the optical densities of the standards, and if the initial dilution of the dye in water is 1 in 50, then plasma volume

$$= \frac{S_1}{T} \times V \times \frac{50}{0.1} \times \frac{8.1}{8.0} \text{ ml.}$$

$$\text{or } = \frac{S_2}{T} \times V \times \frac{50}{0.2} \times \frac{8.2}{8.0} \text{ ml.}$$

Experimental Results

Unless otherwise stated, the procedure was that already described under " Method."

Duration of Shaking Required for Maximum Extraction.—Quantities of plasma, each of 1 ml., were added to a series of round-bottomed centrifuge tubes. To some of these exactly 0.1 ml. of a solution of Evans blue was added. Then 7 ml. of extraction reagent was added to all tubes, which were stoppered and shaken. Tubes containing dye were removed in pairs after varying intervals of time together with one of the tubes containing undyed plasma. Using the latter as a blank the optical density of the dye solution was determined. Fig. 1 shows the variation of extraction with time. Extraction was maximal after eight minutes; the degree of extraction after shaking for five minutes was only very slightly less.

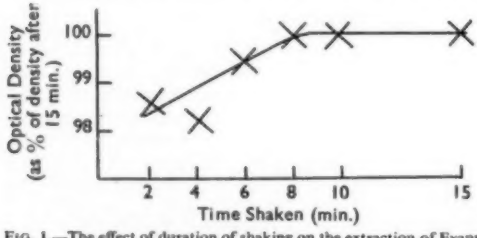

FIG. 1.—The effect of duration of shaking on the extraction of Evans blue.

Consistency of Extraction.—Replicate extractions were made of three different solutions of Evans blue in plasma. The results are shown in Table I. The mean optical densities of the extracted solutions were 0.613, 0.318, and 0.169.

TABLE I
CONSISTENCY OF EXTRACTION OF T1824 FROM PLASMA

Experiment	No. of Replicates	Mean Optical Density	Standard Deviation	Coefficient of Variation (%)
1	7	0·613	±0·004	0·7
2	16	0·318	±0·003	1·0
3	7	0·169	±0·003	1·8

The standard deviations of the means were 0.004, 0.003, and 0.003 respectively. In plasma volume estimations the optical densities encountered fall within the range of 0.100 to 0.400 if doses of 12 to 20 mg. of dye are used in adults.

Efficiency of Extraction.—The extraction of a series of dye dilutions in plasma was compared with a series in which the extraction reagent was added to the same dilutions of dye in distilled water. All extractions were in duplicate, and the results are shown in Table II. The degree of extraction was 96%, or more, in all cases. No allowance is made for the space occupied by protein, since this would affect the results by less than 1%.

TABLE II
EFFICIENCY OF EXTRACTION OF T1824 FROM PLASMA COMPARED WITH WATER

Approximate Concentration of Dye (mg./100 ml. Final Solution)	Optical Density (mμ)				% Extraction
	Plasma Duplicates	Water Duplicates	Mean of Plasma Duplicates	Mean of Water Duplicates	
0·03	0·077 0·078	0·078 0·076	0·0775	0·077	101
0·06	0·166 0·165	0·167 0·169	0·1655	0·168	98·5
0·10	0·302 0·306	0·308 0·312	0·304	0·310	98
0·10	0·312 0·312	0·319 0·319	0·312	0·319	98
0·12	0·330 0·328	0·341 0·342	0·329	0·3415	96
0·16	0·446 0·450	0·462 0·460	0·448	0·461	97
0·20	0·611 0·608	0·622 0·627	0·6095	0·6245	97·5

A similar comparison was made with standard solutions in plasma and distilled water during routine plasma volume estimations. In 15 consecutive estimations the mean percentage extraction was 97.6 (standard deviation ±1.4).

Correction for Haemoglobin in Either Dyed or Undyed Plasma.—Haemoglobin is extracted as acid haematin, which has an absorption peak at 400 mμ, at which wavelength light absorption by Evans blue (T1824) is small (Fig. 2).

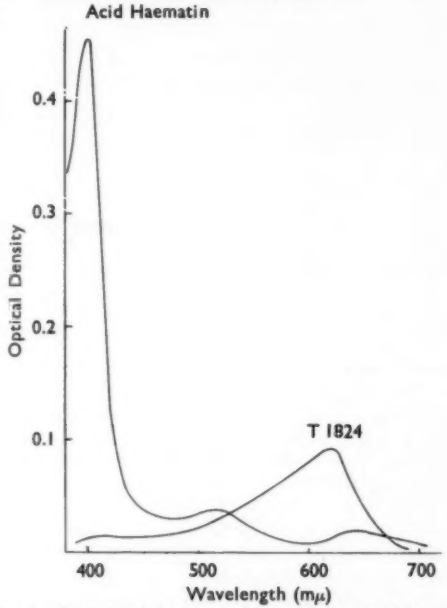

FIG. 2.—The spectral absorption of Evans blue and acid haematin in the extraction reagent.

When 7 ml. of extraction reagent was added to 1 ml. of solutions of haemoglobin in plasma it was found that the resulting acid haematin solutions obeyed the Beer–Lambert law at 400 mμ and 620 mμ for original plasma haemoglobin concentrations of less than 100 mg./100 ml. Above this concentration there was a departure from the straight line relationship. One hundred milligrams haemoglobin/100 ml. plasma represents considerable haemolysis. In over 400 estimations of plasma volume, plasma haemoglobin concentrations were less than 30 mg./100 ml. in all except one case.

A factor for correcting for the presence of haemoglobin was obtained by determining the ratio $\frac{\text{optical density at 400 m}\mu}{\text{optical density at 620 m}\mu}$ for different plasma haemoglobin concentrations as follows.

A large volume of undyed plasma was extracted in the usual manner. To 8 ml. aliquots of the supernatant fluid was added 0.1 ml. of different haemoglobin solutions. The tubes were shaken and centrifuged, and the optical density measured at both wavelengths using a blank consisting of 8 ml. of supernatant fluid and 0.1 ml. of distilled water. The optical depth was 4 cm. at 620 mμ and 1 cm. at 400 mμ. The densities at 620 mμ were therefore divided by 4 when calculating the ratio.

In a similar manner 0.1 ml. quantities of haemoglobin were added to 1 ml. of plasma and extracted as before. The results obtained in these two experiments were substantially the same, and those obtained in the second experiment are shown in Table III.

It is seen that the ratio varies from 28 to 38, the mean value being 35. The largest variations from the mean occur with the lowest haemoglobin concentrations, when small absolute errors have relatively large effects on the ratio, but where the correction for haemoglobin is very small.

In the above experiments, if the observed optical density at 400 mμ is divided by the ratio, the value obtained represents the optical density due to acid haematin at 620 mμ. If the mean ratio of 35 is used for this calculation, the results agree closely with the observed values at 620 mμ. Even in those cases where the experimentally determined ratio differs greatly from the mean, the value obtained by dividing by 35 differs from the observed optical density at 620 mμ by less than 0.002, which is the precision of the photometric readings.

Correction for haemolysis during Evans blue estimation may therefore be made in the manner previously described in the procedure for measurement of plasma volume. For the greatest accuracy, allowance should be made for the absorption due to Evans blue at 400 mμ. This value is very small, however, and ignoring it results in an error in the

TABLE III

DETERMINATION OF THE RATIO $\frac{\text{OPTICAL DENSITY AT 400 m}\mu}{\text{OPTICAL DENSITY AT 620 m}\mu}$ FOR Hb IN PLASMA

Hb concentration (mg./100 ml.)	9·6	19·2	38·4	45	76·8	115	144	153·6	180	192
Density, 400 mμ, 1 cm.	0·064	0·135	0·258	0·320	0·524	0·778	0·957	1·019	1·178	1·239
,, 620 ,, 1 ,, ...	0·0023	0·0045	0·00775	0·0085	0·0152	0·0215	0·0278	0 0298	0·0335	0·0367
Ratio D400/D620	28	30	33	38	35	36	35	34	35	34

corrected optical density at 620 mμ of less than 0.001.

The accuracy of this correction factor is shown in Table IV. Replicate extractions were made of 1 ml. aliquots of dyed plasma. Three of the tubes also contained different amounts of haemoglobin and two others contained distilled water so that the final volume was the same in all tubes. The optical densities were all determined against the same plasma blank. After correction, the haemolysed samples gave a value for Evans blue which is virtually identical with unhaemolysed samples.

TABLE IV

CORRECTION FOR PRESENCE OF HAEMOGLOBIN IN DYED PLASMA

Tube:	1	2	3	4	5
Haemoglobin solution (ml.)	0	0	0·1	0·1	0·1
Distilled water (ml.)	0·1	0·1	0	0	0
Observed optical density at 620 mμ	0·118	0·115	0·124	0·136	0·151
Corrected optical density at 620 mμ	0·118	0·115	0·118	0·117	0·115

Turbidity in Plasma Extracts.—In earlier experiments clear extracts were always obtained even when the filtered extraction reagent was not absolutely clear. In later experiments solutions frequently became turbid after centrifugation. This was found to be due to a rise in temperature of the centrifuge. Extracts always became turbid on standing if the temperature of the centrifuge bucket rose more than 5° C. above room temperature. This did not occur if the temperature in the centrifuge bucket did not rise or if the tubes were cooled in ice for five minutes before centrifuging.

Reproducibility of the Method *in vivo*.—The plasma volume was measured twice in 10 subjects with an interval of 10 minutes between the two measurements. The subjects were healthy students and members of the laboratory staff. All estimations were made in the laboratory with the subjects sitting for the duration of the test.

A sample of blood was withdrawn and 5 ml. of a 0.25% or 0.4% solution of Evans blue was injected through the same needle. Ten minutes later a second sample of blood was withdrawn from a different vein and a second dose of dye injected through the same needle. A third sample of blood was withdrawn from another vein 10 minutes later. The plasma volumes were obtained from the concentrations of dye in the second and third samples described above. The haematocrit was determined on each specimen to assess changes during the test. No significant change occurred.

The results are shown in Table V. It is seen that the difference between the two estimations is less than 2.5% in all cases. In seven cases the second estimation is greater than the first. This difference is significant ($t=2.711$; $P<0.05$), and is due to the fact that the second specimen is used as a blank for the second estimation.

TABLE V

REPRODUCIBILITY OF PLASMA VOLUME ESTIMATION IN NORMAL SUBJECTS

Subject	Plasma Volume 1 (ml.)	Plasma Volume 2 (ml.)	Difference (ml.)
1	2,430	2,460	+30
2	3,900	3,960	+60
3	2,920	2,880	−40
4	2,860	2,890	+30
5	3,730	3,780	+50
6	3,770	3,780	+10
7	2,980	3,050	+70
8	2,960	2,960	0
9	3,500	3,510	+10
10	2,870	2,900	+30

Approximately 1.5% of the dye first injected leaves the circulation in the second 10 minutes (Gibson and Evans, 1937). This leads to a false low reading for the dye concentration in the third specimen and hence a slight overestimate of plasma volume.

Discussion

Crooke and Morris (1942) claimed that extraction of dye was 100%, but reported the results of only one experiment. Reeve (Grant and Reeve, 1951) obtained over 90% extraction in 70 out of 75 estimations. We found in early experiments that extraction was often irregular due to precipitated protein sticking to the wall of the centrifuge tube. Since adopting the practice of shaking the tubes vigorously by hand immediately after the addition of the extraction reagent, extraction has been consistently greater than 95%. This compares well with the value of 97% achieved by Allen (1951) and by Bedwell *et al.* (1955), using chromatographic techniques. Extraction is consistent over a wide range of concentrations. Errors due to incomplete extraction are minimized by the use of standards prepared in the manner described. Certain photochemical effects were noticed by Crooke and Morris (1942) and Morris (1944b). The extraction reagent was found to turn blue after exposure to daylight for 48 hours, and certain batches of phosphotungstic acid were found to decolorize Evans blue. Reeve (Grant and Reeve, 1951) carried out extractions with very little exposure to light and encountered neither of these effects. This has also been our experience. Exposure to

direct sunlight has been avoided and the extraction reagent has been used within 12 hours of preparation. The optical densities of the standards have shown very little variation in several hundred plasma volume estimations, so that decolorization is most unlikely.

Morris (1944b) stated that occasional plasma samples did not yield an absolutely clear supernatant fluid after precipitation. This was uncommon but unpredictable. Grant and Reeve (1951) found that careful handling and sufficient centrifuging always resulted in clear solutions. In the present investigation clear extracts were always obtained if the temperature in the centrifuge bucket did not rise during the centrifuging. It appears that at temperatures above about 20° C. some substance dissolves in the reagent and precipitates on cooling.

The optical correction for the error due to haemolysis described by Morris (1944a) yielded comparable values for extinction due to Evans blue, if the dye was added to extracts of plasma containing varying amounts of haemoglobin. However, the presence of haemolysis has been considered a disadvantage of this method (Morris, 1944b). The spectrophotometric correction described here is simple and gives good results.

The method has been found to be unsatisfactory in a few cases with plasma bilirubin concentrations of 10 to 20 mg./100 ml. Alteration in the nature of this pigment results in a second absorption peak in the red end of the spectrum so that high blank values are obtained. The optical densities of duplicate extractions of deeply jaundiced plasma may sometimes differ widely.

Summary

The extraction of Evans blue from plasma using the hydrochloric - acid - ethanol - phosphotungstic acid reagent of Crooke and Morris has been investigated.

Minor modifications of the procedure for shaking and centrifuging are described. The optical density of the extracted solutions is determined spectrophotometrically using an optical depth of 4 cm. A simple spectrophotometric correction for the presence of haemolysis is described.

With the technique described extraction is consistent and greater than 95%, and turbidity of the extracted solutions is avoided.

Duplicate plasma volume estimations agree within 2.5%.

Various criticisms of the method are discussed.

We wish to thank Dr. G. K. McGowan for his advice and criticism during this work and for his help in the preparation of this paper. We thank Mr. E. Lloyd for preparing the solutions of Evans blue.

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THE MEASUREMENT OF AMMONIA IN WHOLE BLOOD

BY

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The recent widespread interest in blood ammonia determinations in normal and abnormal clinical states (McDermott and Adams, 1954; Schwartz, Phillips, Gabuzda, and Davidson, 1953; White, Phear, Summerskill, and Sherlock, 1955), together with discrepant reports from various laboratories, have prompted a critical examination of the Conway microdiffusion technique in blood analysis.

Method

The method adopted was essentially that described by Conway (1950) with the following modifications. No. 2 units were used with N/4000 $Ba(OH)_2$ in the burette, 0.1 ml. N/2000 HCl in the centre compartment of the unit, and 0.2 ml. saturated K_2CO_3 and 0.2 ml. standard solution or blood in the outer compartment. The measuring error was estimated at 5%. In order to avoid difficulties with the end-point, the blood/carbonate mixture was removed by water pump aspiration immediately before titrating the residual acid.

In this series of analyses blood was withdrawn from the patient with a 5 ml. syringe and No. 1 needle without venous stasis, collected into a bottle containing 50 units of dried heparin, rapidly mixed, and samples withdrawn for analysis two minutes after the beginning of the venipuncture. With practice this could be timed very accurately.

No significant difference was found between heparinized blood and fresh unclotted samples. In some experiments ammonium sulphate was added to the blood using the stock ammonium sulphate solution (100 μg. NH_3-N per ml.) in microlitre quantities, thereby producing negligible dilution of the blood samples.

Results

Serial estimations of the ammonia in standard ammonium sulphate solutions, in samples of freshly shed heparinized normal blood, and in heparinized normal blood, to which measured quantities of ammonium sulphate had been added, were made after given intervals of incubation at room temperature (23° C.).

These results are shown in Figs. 1–4. It is interesting to note that, when the difference between the curves for normal blood and that for blood plus added ammonia is plotted, a very accurate estimate of the added ammonia can be obtained.

Estimations of the ammonia content of blood from cirrhotic patients were made in the same manner. Two typical curves are shown (Fig. 5).

Discussion

The following interpretation for the results is suggested. The ammonia determined in blood under these experimental conditions is made up of two parts: (1) Pre-formed ammonia in the blood sample; (2) ammonia produced during the experiment by the deaminating action of the potassium carbonate.

That pre-formed ammonia can be accurately estimated in blood is demonstrated in those experiments in which ammonium sulphate has been added to normal blood. The plotted recovery curves compare favourably with the curves of ammonia from the standard solutions. However, the amount of ammonia found in blood continues to rise as the incubation period is prolonged. This rise is almost certainly due to the slow production of ammonia by alkaline deamination and not to delayed liberation of pre-formed ammonia.

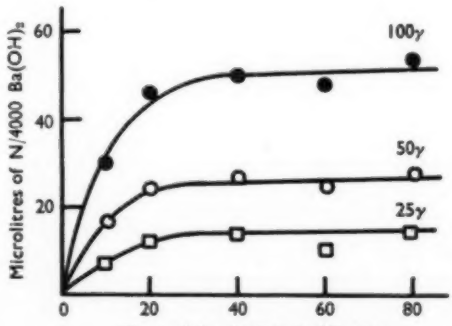

FIG. 1.—Recovery of ammonia from standard ammonium sulphate solutions containing 100, 50, and 25 μg. NH_3-N per 100 ml. The ordinate represents the amount of HCl (expressed in terms of $Ba(OH)_2$) neutralized by the liberated ammonia.

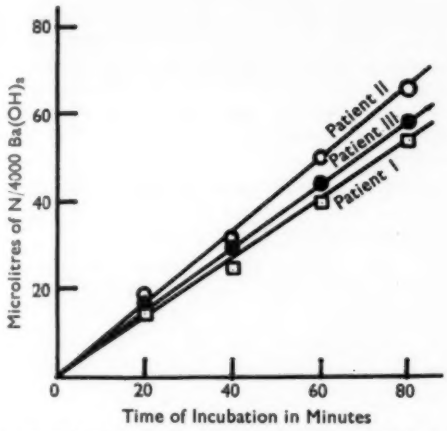

FIG. 2.—Recovery of ammonia from heparinized blood (shed two minutes) incubated at room temperature (23° C.) for varying periods.

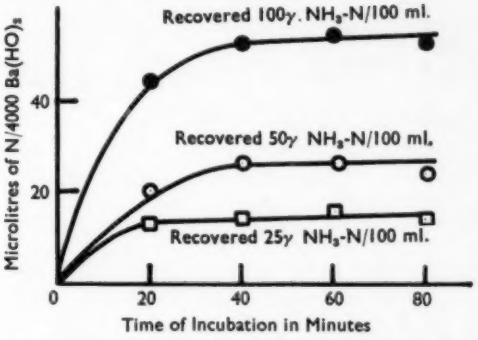
Microlitres of N/4000 Ba(HO)₂
Recovered 100γ NH₃-N/100 ml.
Recovered 50γ NH₃-N/100 ml.
Recovered 25γ NH₃-N/100 ml.
Time of Incubation in Minutes

FIG. 4.—Recovery of ammonia added to heparinized blood. These curves represent the difference between the recoveries shown in Figs. 2 and 3.

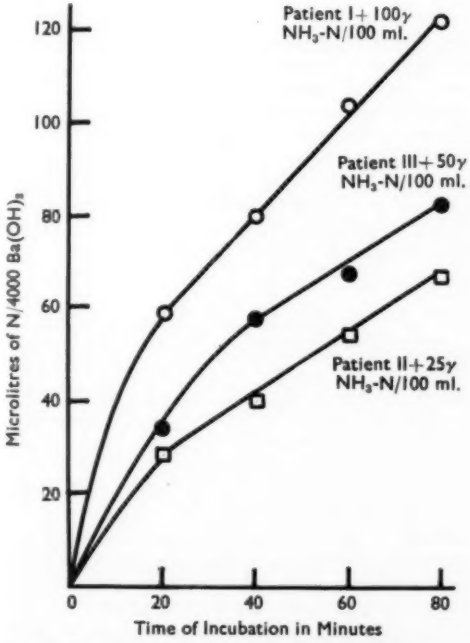
Microlitres of N/4000 Ba(OH)₂
Patient I + 100γ NH₃-N/100 ml.
Patient III + 50γ NH₃-N/100 ml.
Patient II + 25γ NH₃-N/100 ml.
Time of Incubation in Minutes

FIG. 3.—Recovery of ammonia from heparinized blood to which measured amounts of ammonium sulphate have been added.

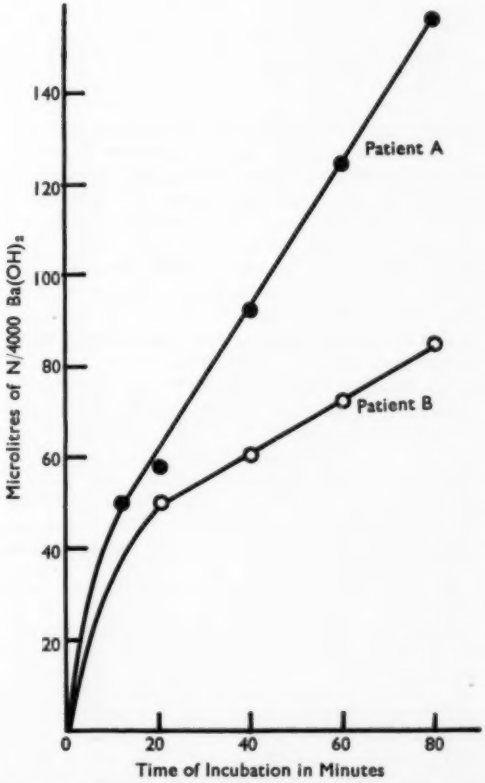

FIG. 5.—Recovery of ammonia from heparinized blood from cirrhotic patients.

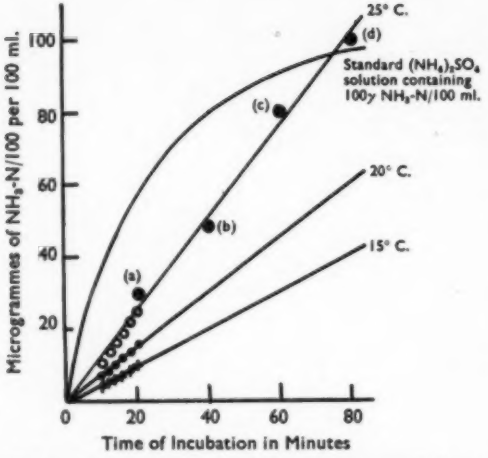

FIG. 6.—Replot of Conway's recovery curve for ammonia from a standard solution together with plots of the correction factors for normal blood at various temperatures (extrapolated). The additional points a, b, c, d, represent the recovery of ammonia from the blood of Patient 1 incubated at 23° C. for varying periods.

Conway made a negative correction for the "special deaminating action of the alkali." He stated that the ammonia formed by the alkali is strictly linear with time over a few hours (Conway, 1935). From his published figures the ammonia liberated by the alkaline deamination of blood had been correlated with the ammonia found in a standard ammonium sulphate solution (Fig. 6). There is a striking similarity with our figures for normal freshly shed blood analysed at room temperature (23° C.). This artefact appears to be immeasurably larger than the pre-formed ammonia in normal freshly shed blood and cannot be disregarded. Should, however, it be constant, a simple correction factor could be applied. Variations were noted with the samples of normal blood evaluated, but these could have been attributed to experimental error ; in the case of abnormal blood, however, this interpretation cannot be sustained. Furthermore, there is no *a priori* indication that the alkali deaminable constituents of abnormal blood will be present in the same concentration as in normal blood.

It is possible to apply individual correction factors to each sample by making the following assumptions:

(*a*) All the pre-formed ammonia in the sample will have been recovered in 40 minutes (within the limits of experimental error).

(*b*) All the ammonia recovered in the subsequent 40 minutes is attributable to deamination.

(*c*) There is no change in the rates of deamination or recovery of ammonia throughout the experiment.

We have no reason to doubt that these assumptions are correct, but further work will be required to verify this modification.

Conclusions

In this brief study of the Conway microdiffusion technique as applied to blood, we have concentrated merely on the artefact produced by alkaline deamination occurring during the period of incubation. We conclude that this artefact forms a disproportionate part of the estimated ammonia, and that whilst Conway's correction factors are confirmed for normal blood these factors cannot be applied indiscriminately to abnormal blood. The amount of pre-formed ammonia in blood is much more accurately measured as the difference between the ammonia liberated in the first 40 minutes and that liberated in the second 40 minutes of incubation ; in normal fresh blood it is extremely small.

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J. clin. Path. (1959), **12**, 131.

SERUM TRANSAMINASE LEVELS IN LIVER DISEASE

BY

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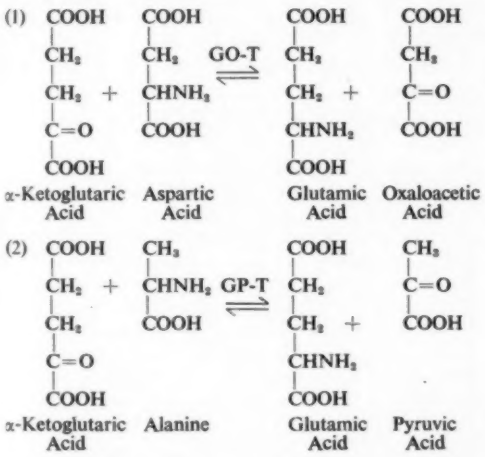
The process of transamination takes place in many tissues in the body and is a fundamental mechanism in the metabolism of amino-acids. Basically it consists of the reversible transfer of an amino group from an amino-acid to a keto acid, providing a link between protein and carbohydrate metabolism.

Although there appear to be a multitude of transamination systems, each catalysed by a specific enzyme, clinical interest centres on the following two reactions.



The first reaction is catalysed by glutamic-oxaloacetic transaminase (GO-T) and the second by glutamic-pyruvic transaminase (GP-T). Measurements are usually made of the serum levels (SGO-T and SGP-T) of these enzymes.

It is presumed that the enzymes are liberated into the circulation during a period of cellular necrosis involving a tissue rich in transaminases. Cardiac muscle has a high content of GO-transaminase, and the high serum levels found in patients with myocardial infarction may be of value in the differential diagnosis of this condition. High levels have also been noted in acute hepatitis (Wróblewski, Jervis, and LaDue, 1956; Madson, Bang, and Iversen, 1958). In addition to its content of GO-T, the liver has a high concentration of GP-transaminase, and serum levels may also be raised in liver disease (Wróblewski and LaDue, 1956). Apart from a slight and inconstant rise in myocardial infarction (cardiac muscle contains much less GP-T than does the liver) these authors failed to detect a rise in SGP-T levels in the absence of liver disease. Therefore parallel determinations of SGO-T and SGP-T may prove to be a sensitive test of liver function. They may also be used as an aid in differential diagnosis, as the relative amounts of the two enzymes vary in differing forms of liver disease (Wróblewski, 1957).

The development of simple methods for the measurement of transaminases in serum has now brought such estimations within the range of the routine laboratory. The present survey was undertaken in order to assess the relationship between the SGO-T and SGP-T levels in patients with liver disease, and to compare these findings with the routine liver function tests.

Methods

Transaminase levels may be measured by incubation of the serum with substrate mixtures of α-ketoglutaric acid and either aspartic acid or alanine. The amount of glutamic acid produced may be determined by quantitative chromatography (Karmen, Wróblewski, and LaDue, 1955), but the method is unsuited to routine work. A spectrophotometric method has been devised by these authors (Karmen, 1955; Wróblewski and LaDue, 1956) whereby the oxaloacetic or pyruvic acids formed are brought into a second enzyme system with reduced diphosphopyridine nucleotide. This reaction is catalysed by the addition of malic (for SGO-T) or lactic (for SGP-T) dehydrogenases with the formation of respectively malic and lactic acids. The rate of disappearance of the oxaloacetic or pyruvic acids is followed on a spectrophotometer. This method requires a suitable instrument and the provision of purified dehydrogenases, but has been used with success by Wróblewski and his colleagues

and has been applied to routine use in Britain (Pryse-Davies and Wilkinson, 1958).

The SGO-T and SGP-T levels in this survey were estimated by a modification of the methods of Cabaud, Leeper, and Wróblewski (1956) and of Reitman and Frankel (1957) reported by Mohun and Cook (1957).

The oxaloacetic acid formed in the reaction is converted to pyruvic acid by aniline citrate. The addition of 2.4 dinitro-phenylhydrazine results in the formation of pyruvic phenylhydrazone which gives a reddish-brown colour when made alkaline.

This method seems to be ideally suited to routine use, as it is simple to perform and requires no special apparatus or reagents.

Measurements were made on a "unicam" S.P. 350 spectrophotometer at 505 mμ, but an EEL portable colorimeter may be used instead. As highly coloured or haemolysed serum may produce falsely high readings, a "serum blank" as recommended by Mohun and Cook (1957) was included in each estimation. The results were converted into the units proposed by Cabaud *et al.* (1956) by a calibration curve described by Mohun and Cook (1957). The upper limits of normal were taken to be 40 units/ml. for both SGO and SGP-T, and an analysis of 100 duplicate estimations showed a standard deviation of 3 units/ml. for levels below 300 units/ml. If levels of over 300 units/ml. were obtained the estimation was repeated using serum diluted with M/15 Sorensen buffer of *p*H 7.5.

The routine liver function tests performed were: Total serum bilirubin (upper limit 1.0 mg./100 ml.), alkaline phosphatase (13 K-A units/100 ml.), thymol turbidity (3 units), and thymol flocculation.

Electrophoretic Mobility

Shepherd and McDonald (1958) reported that the SGO transaminase moved with the α_2-globulin, and an attempt was made to separate the SGO and SGP enzymes on a basis of differing electrophoretic mobility on paper. In five cases of infective hepatitis the initial activity of the serum was high enough to enable this to be done.

Serum, 0.1 ml., was subjected to electrophoresis on 3MM Whatman paper and the run was performed in triplicate. When the albumin had moved about 3–4 in., two of the strips were cut into ½ in. sections which were eluted into M/15 Sorensen buffer *p*H 7.5 and assayed for GO- and GP-T activity respectively. The third strip was stained to show the position of the protein fractions.

The results of two such estimations are shown in Fig. 1. In all five cases there was a clear-cut difference between the electrophoretic mobilities of the two enzymes. The GP-transaminase moves with or just behind the β-globulin while the GO-T moves with the α_2-globulin or is intermediate in mobility between the α_2- and β-globulins. This confirms similar observations by Pryse-Davies and Wilkinson (1958).

Results

Specimens of blood passing through the laboratory for routine liver function tests were all examined for transaminase activity, and cases were noted where either or both of these were abnormal. In this way a total of 64 cases was selected, comprising 16 patients with acute hepatitis, 25 with obstructive jaundice, 17 with chronic liver disease, and six with hepatic metastases. The results of the routine liver function tests and transaminase estimations in each of these groups are shown in Tables I–IV.

Acute Hepatitis.—It is in this condition that the highest levels of transaminase activity are found. The liver has a high content of both enzymes, which are liberated during the phase of active hepatocellular damage. Examination of homogenates of liver from post-mortem specimens showed that the concentration of GP-T was greater than that of the GO enzyme (this result is in conflict with those of Wróblewski and LaDue (1956), who report less GP-T than GO-T in liver). Thus, in cases of acute hepatitis, the SGP-T level might be expected to exceed the SGO-T and this is in fact observed in all the cases examined.

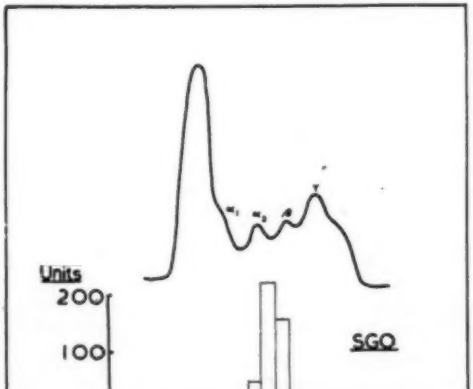


FIG. 1.—Case H.H. (Table I). Total SGO-T = 394 units/ml. Total SGP-T = 1,500 units/ml.

TABLE I

SGO-T AND SGP-T LEVELS IN PATIENTS WITH ACUTE HEPATITIS

Patient	Days from Onset	Bilirubin (mg./100 ml.)	Alkaline Phosphatase (u./100 ml.)	Thymol Turbidity	Thymol Flocculation	SGO-T (u./ml.)	SGP-T (u./ml.)
J.K.	1	2·5	19	7	+	460	2,060
	3	—	—	—	—	500	1,760
	15	1·4	23·5	8·3	+	30	200
	23	0·5	18	6·3	+	13	36
	35	0·5	16	3	Neg.	15	25
S.K.	2	9·0	15	2	,,	620	2,600
	6	10·2	16	4·1	,,	1,000	3,300
	16	5·6	18	2·5	,,	480	1,000
	23	3·1	14	1·6	,,	190	615
	30	3·0	10	1·6	,,	38	114
	37	1·0	12	1·3	,,	22	27
B.R.	1	—	—	—	—	Over 4,000	Over 4,000
	3	2·4	—	—	—	1,550	3,000
	10	6·8	20	1	Neg.	120	420
	23	1·2	7·5	1	,,	19	5
	40	1·0				18	24
D.B.	7	9·0	22	5·6	,,	400	3,500
H.R.	14	0·6	11	2	,,	43	108
G.D.	7	6·3	21·5	7	+	1,800	1,600
H.H.	9	4·5	26	4·7	Neg.	394	1,500
R.G.		4·6	23	1	,,	29	55
D.M.	8	4·1	31	1·5	,,	210	370
D.F.	26	3·2	6·0	1	,,	10	9
J.P.R.	50	2·2	13·5	1	,,	20	30
D.O.	17	0·8	8	1	,,	27	89
S.B.	3	1·4	10·5	2·5	,,	80	380
C.A.	14	1·2	19	1	,,	80	860
P.S.*	6	1·6	48	6	+	110	350
R.M.*		0·7	19	5	+	15	44

* Acute hepatitis complicating glandular fever.

The serum levels of both enzymes will rise until the cellular necrosis is at its maximum and will thereafter fall to normal limits. The level at any particular time will therefore be governed (*a*) by the amount of active necrosis causing a fresh release of enzyme, and (*b*) by the normal processes of globulin metabolism which will remove the enzyme from the circulation. When the acute phase is over, only (*b*) will operate, but if the liver damage continues an equilibrium will be attained and the serum level of the enzymes will remain elevated. This is presumably the case in patients with subacute hepatitis (Cases S. U., A. P., and G. S. in Table III).

In the cases shown in Figs. 2 and 3 the steady clinical improvement after the icteric phase and the gradual decline in the liver function tests to normal led us to postulate that the period of necrosis was not prolonged. The pattern of decline of the serum transaminase activity in these cases was of interest, as it afforded a measure of the rate of metabolism of the α- and β-globulins. If the levels are plotted against time on a semi-logarithmic scale the rate of decay is approximately the same for both enzymes in each case. The survival time of the biologically active molecules lies between 11 and 24 days, and from the slope of degradation the half life is of the order of seven to 10 days. This is roughly in line with the existing estimate (Martin, 1957) of a half life of 10 days for pseudo-cholinesterase, another of the α_2-globulins (Kekwick, 1955).

Fig. 4 shows the general relationship between the duration of the disease and the serum transaminase levels in 14 cases of acute hepatitis. In the two cases shown graphically (Figs. 2 and 3), there was a close correlation between the transaminase levels and routine liver function tests, but this was not so in every case. In two patients (H. R. and D. O., Table I), the routine tests were normal, but the raised transaminase levels presumably indicated that the period of activity of the disease had been prolonged, and in four cases (J. K., D. F., J. P. R., and B. R., Table I) the liver function tests were abnormal in the presence of normal transaminases. This makes it doubtful if the latter can be considered in isolation in assessing the progress of acute hepatitis. However, SGO-T determinations were used by O'Brien, Goble, and MacKay (1958) to assess the effectiveness of cortisone in chronic hepatitis. The findings in acute hepatitis complicating glandular fever are similar to those in infective hepatitis.

TABLE II

SGO-T AND SGP-T LEVELS IN PATIENTS WITH OBSTRUCTIVE JAUNDICE

Patient	Bilirubin (mg./100 ml.)	Alkaline Phosphatase (u./100 ml.)	Thymol Turbidity	Thymol Flocculation	SGO-T (u./ml.)	SGP-T (u./ml.)
G.H.	13·0	30	1	Neg.	45	71
A.F.	8·4	86	1	,,	64	300
W.H.	1·0	46	1	,,	210	560
	0·5	15	1	,,	66	300
A.D.	11·4	38	1	,,	33	47
K.G.	3·5	30	1·2	,,	107	Over 300
E.S.	1·6	16	1·2	,,	60	83
M.G.	17·5	31·5	1·5	,,	69	62
F.C.	1·9	31·5	1	,,	80	120
A.I.	8·5	61	1	,,	27	72
E.H.	0·5	23·5	1	,,	24	13
R.G.	12·3	34·5	1	,,	32	43
S.N.	2·8	65	1	,,	46	48
H.F.	2·0	56	1	,,	220	720
J.B.	0·5	18	1	,,	48	112
P.C.	11·5	8·5	1·7	,,	30	60
M.M.	4·2	133	1	,,	62	102
J.L.	1·7	83	1·5	,,	59	83
S.W.	9·0	39	1	,,	2	6
S.F.	1·1	22	1	,,	24	375
N.W.	0·9	13·5	1	,,	470	990
A.K.	2·7	13·5	1	,,	58	300
B.P.	3·6	25	3·8	,,	140	360
S.M.*	1·2	18	1	,,	38	155
W.S.*	3·2	27	1	,,	111	200
B.S.†	11·1	39	1·5	,,	93	97

* Cases of jaundice complicating chlorpromazine therapy.
† Intrahepatic biliary obstruction.

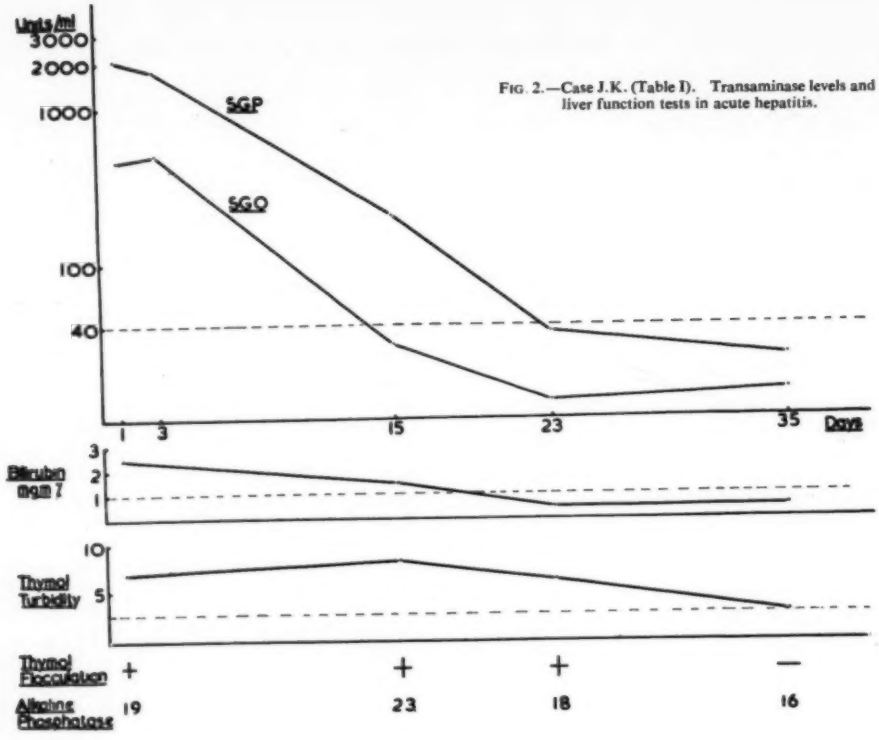


FIG. 2.—Case J.K. (Table I). Transaminase levels and liver function tests in acute hepatitis.

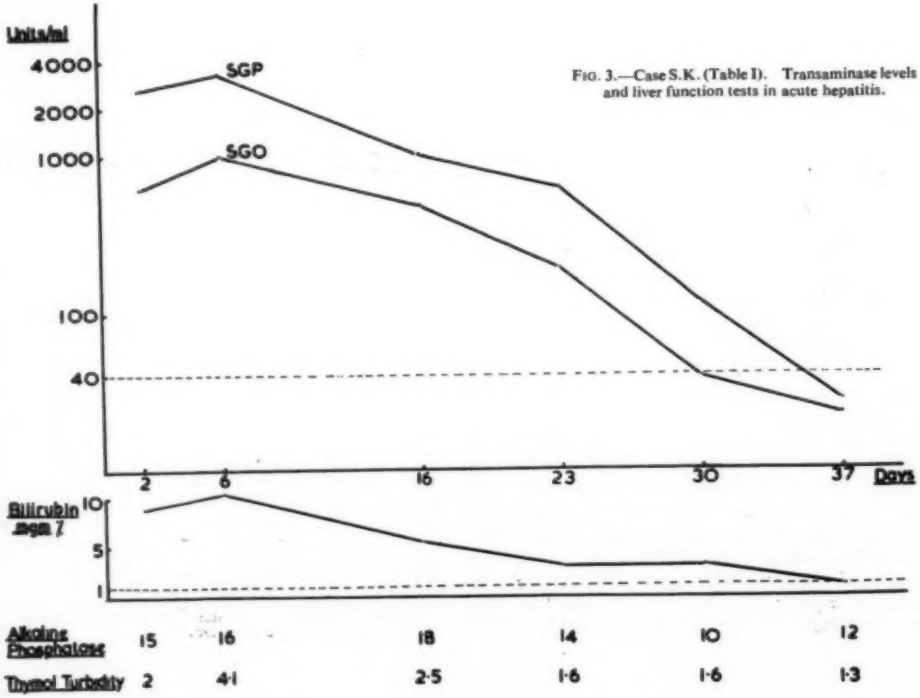


FIG. 3.—Case S.K. (Table I). Transaminase levels and liver function tests in acute hepatitis.

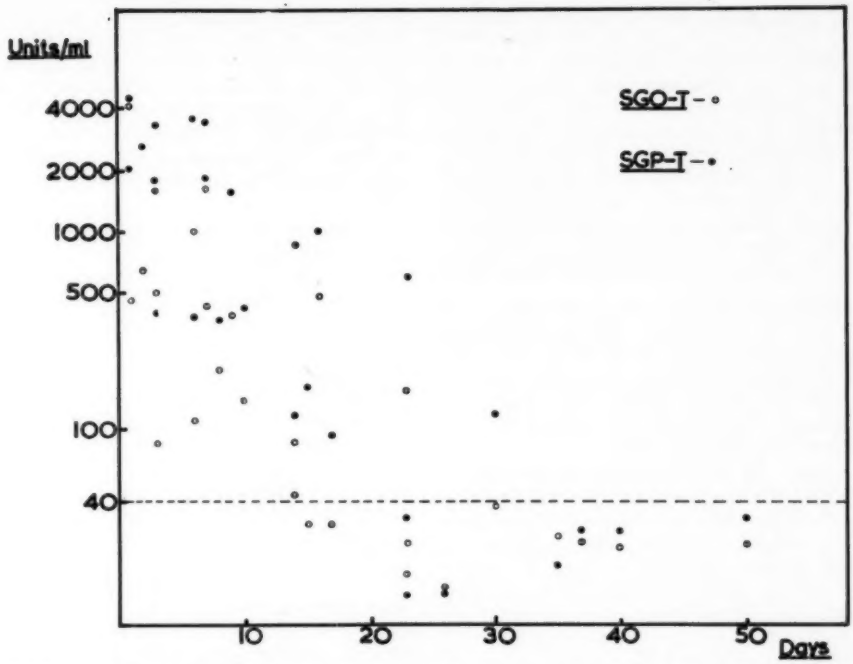


FIG. 4.—The relationship between the duration of the disease and the serum transaminase levels in 14 cases of acute hepatitis.

Obstructive Jaundice.—Twenty-five cases of obstructive jaundice were studied (Table II) and in only two cases (E. H. and S. W.) were the transaminase levels within normal limits. In all the other cases the SGP-T level was raised, this being associated with a smaller rise in SGO-T levels in 16 cases and a normal SGO-T level in the remaining six. In only one case (M. G.) did the SGO-T exceed the SGP-T level.

The liver function tests showed a constant pattern, namely, a raised bilirubin and alkaline phosphatase level with a normal thymol turbidity and flocculation reaction. The extent to which the transaminase levels were raised bore no constant relationship to the degree of abnormality of the routine tests. The one case (N. W.) in which these were normal showed extremely high transaminase levels and was judged clinically to have had an attack of biliary colic associated with gall stones.

The two cases of jaundice complicating largactil therapy are included in this group, as both the clinical and biochemical findings in such cases are similar to those in patients with extrahepatic biliary obstruction.

TABLE III

SGO-T AND SGP-T LEVELS IN PATIENTS WITH CHRONIC LIVER DISEASE

Patient	Bilirubin (mg./100 ml.)	Alkaline Phosphatase (u./100 ml.)	Thymol Turbidity	Thymol Flocculation	SGO-T (u./ml.)	SGP-T (u./ml.)
D.A.D.	1·4	12	1	Neg.	43	136
J.S.	1·8	7·5	3·7	,,	175	44
G.S.M.	0·5	6	1	,,	44	22
L.H.	1·4	18	2	,,	14	5
E.S.	0·5	17	1	,,	40	22
L.P.	0·5	13·5	1·5	,,	68	63
A.N.	2·0	24	7	+	67	18
C.O.	0·6	26·5	1	Neg.	77	21
W.B.	1·8	19·5	12	+	110	140
G.A.S.	3·4	50	3·6	+	110	113
E.W.	0·5	12	1	Neg.	47	78
G.A.	0·5	10	1·2	,,	63	54
S.U.*	0·7	7·5	2·5	+	41	87
A.P.*	1·6	13	16	+	120	170
G.S.*	2·4	38	3	Neg.	73	123
A.S.†	2·6	11·5	1	,,	67	152
R.A.†	1·2	11	9	+	115	300

* Subacute progressive hepatitis. † Acute-on-chronic hepatic disease.

Chronic Liver Disease.—The results are listed in Table III. The pattern of both the liver function tests and transaminase levels was variable. It would seem that in the subacute

(Cases U.V., A.P., and G.S.) and acute-on-chronic (Cases A.S. and R.A.) types both transaminases are raised, the SGP-T showing the greater increase. This would be consistent with a process of active liver cell damage. Of the 12 patients with established chronic liver damage, the serum levels of both enzymes were normal in two, the SGO-T was greater than SGP-T in five cases, the SGP-T being greater in the other five cases. There was no constant relationship to the routine tests, but a high thymol turbidity value seems often to be associated with a raised SGO-T level.

Secondary Carcinoma.—In these cases, the routine tests usually reveal a rise in the alkaline phosphatase level, and Table IV shows that in six cases with hepatic metastases this was a constant feature. In all cases the SGP-T level was raised and was higher than the SGO-T level in the four cases where this was also raised.

In patients with skeletal metastases the alkaline phosphatase may be raised, but the transaminase levels will be normal. In one such patient with secondary deposits in bone but none in the liver the alkaline phosphatase was 91 units/100 ml. and the SGO and SGP-T levels were 13 and 6 units/ml. respectively.

TABLE IV

SGO-T AND SGP-T LEVELS IN PATIENTS WITH HEPATIC METASTASES

Patient	Bilirubin (mg./100 ml.)	Alkaline Phosphatase (u./100 ml.)	Thymol Turbidity	Thymol Flocculation	SGO-T (u./ml.)	SGP-T (u./ml.)
H.M.	6	52	1	Neg.	37	63
W.R.	0·5	27	1	,,	45	62
J.G.	0·5	36	0·8	,,	80	125
J.H.	4·2	35	1	,,	35	97
C.A.	0·9	25	1	,,	56	144
S.C.	2·3	46	1	,,	200	560

Discussion

The method of Mohun and Cook (1957) was found to work very well in practice and can be carried out easily in a routine laboratory. On the whole the results obtained agree with those of Wróblewski (1957) and Pryse-Davies and Wilkinson (1958) using the spectrophotometric method.

In acute hepatitis the high levels of transaminase in the serum make the estimations a very sensitive test of liver function, the SGP-T levels being higher than the SGO-T in the acute phase. Serial assays are useful in following the progress of the disease, but the levels may not necessarily be a more sensitive index than the routine tests. We would agree with Wróblewski (1957) when he states that transaminase estimations should not be considered in isolation but when taken in conjunction with other biochemical and clinical findings may prove to be of value. A persistently raised transaminase level will presumably indicate continuing liver cell damage.

In chronic liver disease the transaminases are not always abnormal, and where they are raised the relationships between the SGO-T and SGP-T levels are so variable that it is doubtful if any firm conclusions can be drawn from them. On the other hand in all his cases of cirrhosis Wróblewski (1957) found a constant rise in the SGO-T which always exceeded the SGP-T level.

The most consistent findings were in cases of obstructive jaundice where almost every case showed a rise in the SGP-T level over that of SGO-T. Pryse-Davies and Wilkinson (1958) found this ratio to be reversed in their series, but our results would confirm those of Wróblewski (1957), whose method was used by the latter authors. Unfortunately the estimations are of the least value in this group, as it is usually easy to diagnose extrahepatic obstructive jaundice on clinical grounds and by routine liver function tests.

In cases of secondary carcinoma we found the SGP-T levels to be consistently elevated, whereas Wróblewski (1957) reports a higher rise in SGO-T levels. However, the number of patients in our series is too small to allow any definite conclusions to be drawn.

Summary

Serum transaminase levels were estimated in 64 cases of liver disease and the results compared with the routine liver function tests. Particular attention was paid to the relation between the serum levels of glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, and determinations of these were found to be a sensitive test of liver function.

The electrophoretic mobilities of the enzymes were confirmed, the GO-transaminase moving with the α_2-globulin and the GP-transaminase with the β-globulin.

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A SOURCE OF ERROR IN SERUM GLUTAMIC-OXALOACETIC TRANSAMINASE DETERMINATION

BY

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In the summer of 1958 we observed that the results of serum glutamic-oxaloacetic transaminase (SGO - T) determinations by the spectrophotometric method of Karmen, Wróblewski, and LaDue (1955) appeared to be appreciably higher than during previous months. This suspicion was confirmed when the sera from three out of six healthy young adults were found to give SGO-T values greater than 40 units per ml., the upper limit of the normal range found in these laboratories by Pryse-Davies and Wilkinson (1958). No increase during this period was noticed in the range of serum glutamic-pyruvic transaminase (SGP-T) figures determined by the similar method of Wróblewski and LaDue (1956).

The spectrophotometric SGO-T and SGP-T methods are based respectively on the following pairs of reactions:

$$\left\{\begin{array}{l}\text{aspartic acid} + \alpha\text{-oxoglutaric acid} \xrightarrow{\text{SGO-T}} \text{oxaloacetic acid} + \text{glutamic acid} \\ \text{oxaloacetic acid} + \text{D.P.N.H.} \xrightarrow{\text{malic dehydrogenase}} \text{malic acid} + \text{D.P.N.}\end{array}\right.$$

$$\left\{\begin{array}{l}\text{alanine} + \alpha\text{-oxoglutaric acid} \xrightarrow{\text{SGP-T}} \text{pyruvic acid} + \text{glutamic acid} \\ \text{pyruvic acid} + \text{D.P.N.H.} \xrightarrow{\text{lactic dehydrogenase}} \text{lactic acid} + \text{D.P.N.}\end{array}\right.$$

In each case the transaminase activity is proportional to the rate of oxidation of reduced diphosphopyridine nucleotide (D.P.N.H.), the destruction of which is followed by observing the fall in optical density at 340 mμ.

Impurity of Malic Dehydrogenase

The increase in SGO-T figures appeared to coincide with the introduction of a new batch of malic dehydrogenase (M.D.H. 1), but this enzyme did not effect transamination in the absence of serum and hence did not appear to be contaminated with a transaminase. The explanation of the anomaly, however, was obtained from studies with pyridoxal 5-phosphate which is known to function as a coenzyme in transamination reactions (Lichstein, Gunsalus, and Umbreit, 1945). Considerable transaminase activity was demonstrated when 0.2 ml. of a solution containing 0.2 μg. pyridoxal 5-phosphate replaced the 0.2 ml. of serum normally used in the SGO-T test. Moreover, the combination of M.D.H. 1 and pyridoxal 5-phosphate was shown to effect transamination between aspartic acid and α-oxoglutaric acid, the formation of glutamic and oxaloacetic acids being demonstrated by paper chromatography. Neither M.D.H. 1 alone nor pyridoxal 5-phosphate alone could produce this transamination, nor were pyridoxine hydrochloride or pyridoxamine dihydrochloride able to replace pyridoxal 5-phosphate.

These results suggested that pyridoxal 5-phosphate activated a glutamic-oxaloacetic apo-transaminase contaminating M.D.H. 1. Malic dehydrogenase is obtained commercially from heart muscle, a tissue rich in glutamic-oxaloacetic transaminase, and it is likely that the end-product might be contaminated by the corresponding apo-transaminase, the coenzyme having been removed during purification. The presence of the contaminating apo-transaminase would remain undetected until the addition of pyridoxal 5-phosphate.

Adding pyridoxal 5-phosphate to lactic dehydrogenase (the enzyme used for SGP-T determination) showed that it too contained glutamic-oxaloacetic apo-transaminase but in a much smaller amount than M.D.H. 1. Lactic dehydrogenase is prepared from skeletal muscle, a tissue which contains glutamic-oxaloacetic transaminase but does not contain a significant amount of glutamic-pyruvic transaminase. Contamination with glutamic-oxaloacetic apo-transaminase and freedom from contamination

with glutamic-pyruvic apo-transaminase is therefore to be expected, and this would explain our inability to demonstrate an effect of pyridoxal 5-phosphate on the glutamic-pyruvic transaminase system.

A second sample of malic dehydrogenase (M.D.H. 2) obtained from a different supplier showed no transaminase activity when pyridoxal 5-phosphate was added in 10 times the concentration previously found to activate M.D.H. 1. Parallel SGO-T determinations were made on a number of sera using both batches of malic dehydrogenase, and the results were consistently 5–10 units lower with the new sample. Varying the concentration of M.D.H. 2 did not affect the SGO-T activity of sera, but similar alterations in the concentration of M.D.H. 1 caused appreciable changes in the apparent SGO-T figures. It may therefore be concluded that the high results obtained with M.D.H. 1 were due to its contamination with a glutamic-oxaloacetic apo-transaminase activated by pyridoxal present in serum.

Experiments with pyridoxal 5-phosphate indicated that the discrepancy between the SGO-T values obtained using the malic dehydrogenases from the different suppliers could be accounted for by assuming a serum pyridoxal 5-phosphate level of between 10 and 100 μg. per 100 ml.

Conclusions

It is recommended that batches of malic dehydrogenase intended for SGO-T determination should not only be tested for possible transaminase contamination using a blank containing malic dehydrogenase and omitting serum, but also for apo-transaminase content using a blank containing pyridoxal 5-phosphate in place of serum. Alternatively new supplies of malic dehydrogenase may be compared with a batch known to give satisfactory results by carrying out parallel SGO-T determinations on a number of different sera, the method used during the course of the study by Pryse-Davies and Wilkinson (1958).

It is further suggested that the activation by pyridoxal 5-phosphate of the glutamic-oxaloacetic apo-transaminase contaminating certain preparations of malic dehydrogenase might form the basis of a method for the determination of pyridoxal.

Summary

Erroneously high serum glutamic-oxaloacetic transaminase (SGO-T) levels determined by the spectrophotometric method were found to be due to the presence of an apo-transaminase in the malic dehydrogenase used.

It is recommended that each batch of malic dehydrogenase should be tested for the presence of this apo-transaminase by carrying out blank determinations in which serum is replaced by pyridoxal 5-phosphate.

The possibility of using samples of malic dehydrogenase containing apo-transaminase as a means of determining pyridoxal is suggested.

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THE INHIBITORY EFFECT OF TRIAMCINOLONE ON ADRENAL FUNCTION

BY

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Many steroid hormones will depress the secretion of hydrocortisone by the adrenal by inhibiting secretion of pituitary corticotrophin. This inhibition has proved a useful means of demonstrating an intact pituitary-adrenal axis, and may help to distinguish between adrenal cortical neoplasia and hyperplasia (Jailer, Gold, and Wallace, 1954; Cope, 1956). However, most of these hormones are of little use for this purpose because suppressive doses contribute significantly to the metabolites whose measurement in urine and plasma is used as an index of adrenal activity. With the introduction of the more potent steroid, 9-alpha-fluoro-hydrocortisone, the dose required to produce adrenal depression was considerably reduced, and the interference with steroid analysis produced by metabolites of the administered hormone was therefore less. The 16-hydroxylated derivative of 9 - alpha - fluoro - hydrocortisone, " triamcinolone " (9 - alpha - fluoro - 16 - alpha - hydroxyl-delta-hydrocortisone acetate), is reported (Bernstein, Lenhard, Allen, Heller, Littell, Stolar, Feldman, and Blank, 1956) to have less glucocorticoid activity than its parent hormone. This suggests that larger doses would be required to produce adrenal suppression (DiRaimondo and Forsham, 1958), which in consequence may interfere with measurement. However, plasma hydrocortisone concentrations in two patients receiving the hormone therapeutically were observed to be well below normal levels. This was particularly surprising in view of the chemical structure of the compound, and led to the present investigations in man of the ability of " triamcinolone " to depress adrenal secretion, and an assessment of its suitability for clinical use in adrenal function tests.

Methods

Plasma hydrocortisone was determined by the method of Peterson, Karrer, and Guerra (1957) with the following modifications.

(1) Methylene dichloride was shaken twice with concentrated sulphuric acid, neutralized with sodium hydroxide, washed four to five times with water, and redistilled.

(2) Phenylhydrazine was dissolved in ethanol and treated with activated charcoal. It was recrystallized after filtration.

(3) The extraction technique was simplified by vigorously shaking the plasma and methylene dichloride for one minute in 8-in. " quickfit " stoppered tubes. No trouble was experienced with emulsions.

Procedures (1) and (2) reduced the reagent blank considerably. The plasma was separated immediately after collection and frozen until analysed. Urinary 17, 21 di-hydro-20-ketosteroids were determined before and after hydrolysis with rat β glucuronidase by the method of Silber and Porter (1954) as modified by Liddle, Richard, and Peterson (1955).

Urinary 17-ketosteroids were measured as recommended by the M.R.C. (1951) and 17-ketogenic steroids and " total 17-hydroxycorticosteroids " were determined by the methods of Norymberski (Norymberski, Stubbs, and West, 1953; Appleby, Gibson, Norymberski, and Stubbs, 1955). All urines were collected without preservative and refrigerated until analysed.

Two subjects, P.T., a girl with cirrhosis without ascites aged 16 years, and C.H., a normal male aged 31 years, were studied in detail. Toxic side-effects of " triamcinolone " permitted only control observations on a third subject, who was therefore excluded from the study. During the observation period, daily 24-hour urine specimens were collected (beginning at 9 a.m.) and blood specimens were obtained at the end of each urine collection interval. After a two-day control period, " triamcinolone " (8 mg., q.d.s.) was administered for 11 and five days respectively. During the last two days of " triamcinolone " administration, 100 i.u. of A.C.T.H. gel (Armour) was given intramuscularly at 9 a.m. Plasma hydrocortisone was measured in blood drawn at 9 a.m. from two cirrhotic patients, who had been receiving " triamcinolone " (8 mg. q.d.s.) for eight days.

Results

The ability of pure " triamcinolone " to react in the above-noted techniques was studied. The

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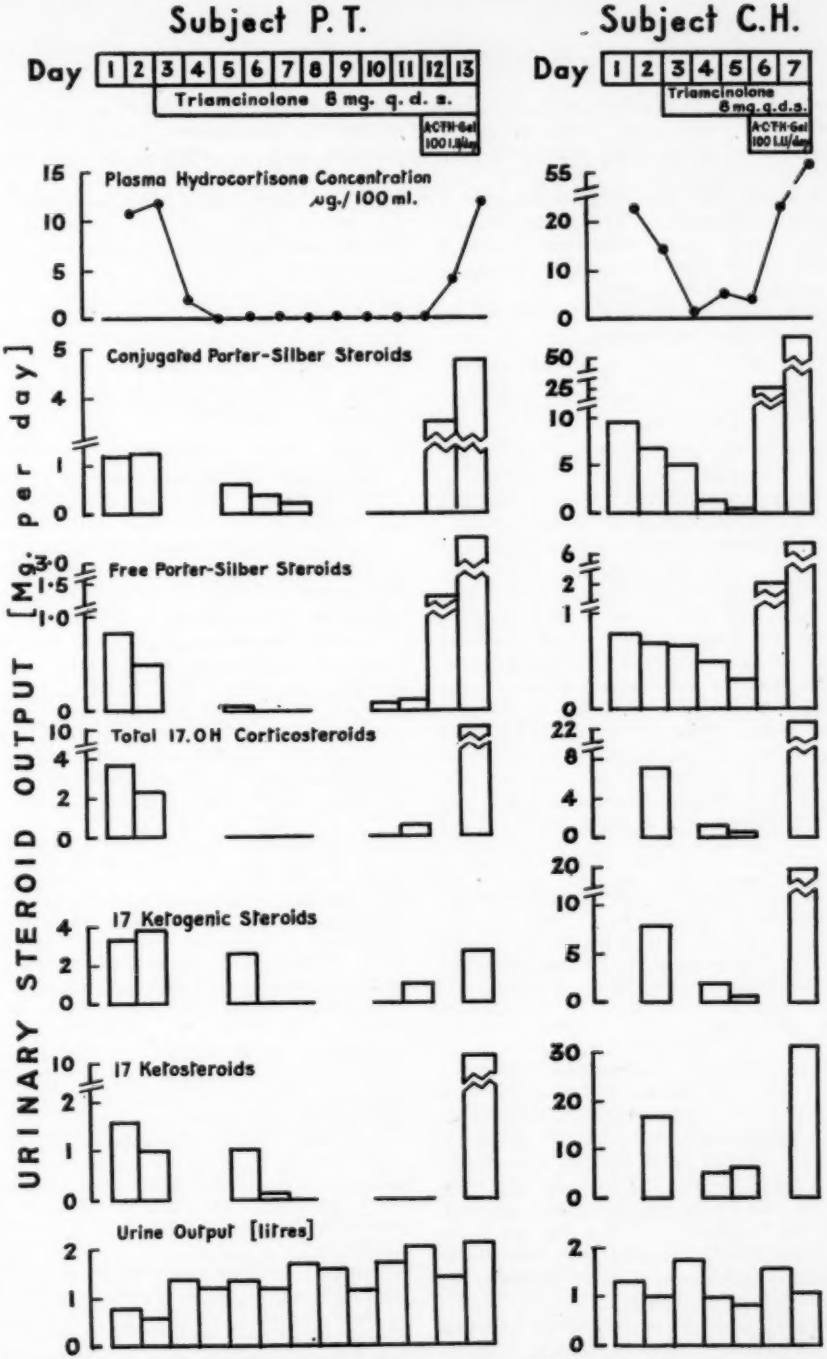
Subject P. T.
Subject C.H.
Day
1 2 3 4 5 6 7 8 9 10 11 12 13
Triamcinolone 8 mg. q. d. s.
A·C·T·H·Gel 100 I.U./day
Day
1 2 3 4 5 6 7
Triamcinolone 8 mg. q. d. s.
A·C·T·H·Gel 100 I.U./day
Plasma Hydrocortisone Concentration µg./100 ml.
Conjugated Porter-Silber Steroids
Free Porter-Silber Steroids
Total 17.OH Corticosteroids
17 Ketogenic Steroids
17 Ketosteroids
Urine Output [litres]
URINARY STEROID OUTPUT [Mg. per day]

FIG. 1.—Plasma hydrocortisone concentrations measured at 9 a.m. and 24-hour urinary corticosteroid outputs measured in two subjects before and during the administration of "triamcinolone" and "triamcinolone" and A.C.T.H. gel.

chromogenicity of " triamcinolone " compared on a weight for weight basis was no greater than 1% of that of dehydroisoandrosterone using the techniques measuring 17-ketosteroids and 17-ketogenic steroids, and 3% for the " total 17-hydroxycorticosteroids." The direct Zimmerman test gave no detectable reaction. Chromogenicity by the Porter-Silber technique was 3% of that of hydrocortisone.

After seven days of " triamcinolone " therapy the two cirrhotic patients had plasma hydrocortisone concentrations of 0.6 and 1.6 γ % respectively at 9 a.m., which is well below the lower limit of normal, i.e., 6–25 μg. %, for the method (Peterson *et al.*, 1957).

The results of the detailed studies on the two subjects are presented in Fig. 1. The plasma hydrocortisone concentration fell to subnormal levels after the administration of " triamcinolone," and a parallel reduction in urinary steroid output was observed. The data further show that suppression was not maximal until after three days of receiving " triamcinolone." Both plasma hydrocortisone concentration and urinary steroid output increased rapidly after A.C.T.H. therapy. In P.T. the daily urinary output during " triamcinolone " administration was consistently higher than output during the whole period, while in C.H. no consistent change in urinary output was observed. It should be noted that the basal steroid excretion in P.T. was low. This was probably associated with the liver disorder, though her age and sex may have been contributory factors.

Toxic effects, consisting of lightheadedness, nausea, and insomnia, made it necessary to discontinue the use of the drug in one subject not reported here. In the other subjects no untoward effects were noted.

Discussion

The observation that " triamcinolone " produces little Porter-Silber chromogen is of considerable interest as this hormone possesses a 17, 21, dihydroxy-20-ketosteroid configuration which is characteristic of Porter-Silber reacting compounds (Silber and Porter, 1954). The hydroxyl group on C 16 may account for the relative inactivity of this steroid in this reaction. Because this hormone does not significantly interfere with commonly used measuring techniques, study of its effects on adrenal function is simplified.

The present findings of markedly reduced plasma hydrocortisone and diminished urinary corticosteroid output indicate adrenal depression during " triamcinolone " administration. The prompt rise of plasma hydrocortisone and urinary steroids following A.C.T.H. gel given simultaneously with " triamcinolone " suggests that this hormone does not impair the adrenal responses to A.C.T.H. It appears, therefore, that " triamcinolone " acts similarly to other adrenal corticoids, such as cortisone, by inhibiting pituitary A.C.T.H. production (Sayers and Sayers, 1948). The delay before the maximal adrenal depression by " triamcinolone " indicates that for clinical testing the steroid should be administered for a period of not less than three days.

Summary

The effect of a new synthetic hormone, " triamcinolone," on adrenal function was investigated.

" Triamcinolone," in the dose employed, did not produce a significant amount of urinary or plasma chromogen with any of the commonly employed techniques for measuring corticosteroids.

Marked depression of adrenal function was found in two subjects studied while they were receiving the drug. The response to A.C.T.H. gel while the subjects were receiving " triamcinolone " indicated that the suppressive effect was not a direct action of the steroid on the adrenals.

It is concluded that " triamcinolone " is suitable for clinical use in tests of adrenal inhibition.

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COMPLEXIMETRIC DETERMINATION OF CALCIUM IN PATHOLOGICAL AND PHYSIOLOGICAL SPECIMENS

BY

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Titrimetric estimation of calcium with disodium ethylene diamine tetra-acetate (E.D.T.A., sodium edetate), as performed on serum (Baron and Bell, 1957), cannot be applied directly to most other biological specimens because of their high concentration of phosphate. A high ratio of phosphate to calcium in the material titrated causes a drawn-out and inaccurate endpoint. A rapid procedure for calcium determination has been devised which gives accurate results after separation of phosphate.

METHOD

Reagents

(1) Deionized or glass-distilled water must be used throughout all stages of the procedure.

(2) **For Ashing.**—N-HCl is used.

(3) **For Phosphate Separation** (Horner, 1955).—(*a*) 48% morpholine nitrate: 50% v/v HNO_3 is slowly added to 28 ml. morpholine at 0° C. until the solution is neutral or slightly acidic to B.D.H. universal indicator paper (about 40 ml. acid is required). The solution is then diluted to 100 ml. with water.

(*b*) The morpholine nitrate-nitric acid reagent is a 1:1 mixture of 48% morpholine nitrate and 25% v/v HNO_3.

(*c*) 66% w/v sodium tungstate $Na_2WO_4 2H_2O$.

(4) **For Titration.**—The following reagents are made up:

(*a*) 40% w/v NaOH.

(*b*) Glycine buffer *p*H 12.66 of 20 ml. of 0.1 *M*-glycine+0.1 *M* NaCl and 80 ml. of 0.1 *M* NaOH.

(*c*) E.D.T.A. solution for which 0.93 g. disodium ethylene diamine tetra-acetate (E.D.T.A.) is dissolved in water and the solution made up to 1,000 ml.

(*d*) *Stock Calcium Solution* (100 mg./100 ml.).—To 2.5 g. $CaCO_3$ A.R. (previously dried at 105° for 24 hours) is added about 200 ml. water followed by 50 ml. N-HCl. After standing overnight the solution is diluted to 1,000 ml.

(*e*) *Calcein-thymolphthalein Indicator.* — Calcein, 0.2 g. (the Hopkin and Williams material has been found suitable), 0.12 g. thymolphthalein, and 20 g. potassium chloride are ground together to a fine powder (Tucker, 1957). This mixture is stable for several months when kept dry.

Preparation of Sample for Phosphate Separation from Original Biological Specimen

Serum/Plasma and Similar Fluids.—No preparation or phosphate separation required, unless the serum is grossly icteric or haemolysed.

Faeces.—The specimen is mixed and diluted to a thin, creamy consistency, of expected calcium concentration 10–30 mg./100 ml. It is usually convenient to homogenize the faeces (with addition of water if necessary), then an aliquot is taken which is equivalent to 1/10 of the daily faecal excretion and this is diluted 1 in 5.

Food.—The food is cut into small pieces and homogenized, adding water if necessary. The approximate calcium content of food is usually known, and it should be diluted so that the expected calcium concentration is about 20 mg./100 ml.

Milk.—The specimen is diluted so that the expected calcium concentration is about 20 mg./100 ml. A dilution of 1 in 5 is normally suitable for cow's milk.

Urine (and Other Fluids).—The calcium concentration of urine varies over a wide range. Very often in clinical work an estimate of the urinary calcium concentration can be obtained by reference to the patient's condition. This is not usually possible in experimental work and then it is advisable to carry out a screening test as described below. The commonly used Sulkowitch test (Barney and Sulkowitch, 1937) for urinary calcium was not found reliable enough for our purposes.

To each of two test-tubes are added 5 ml. of glycine buffer, 1 ml. of E.D.T.A., and 0.5–1 mg. of calcein-thymolphthalein indicator. Then 2 ml. urine is added to the first tube and 0.2 ml. to the second tube. The calcium concentration of the urine may then be estimated by viewing in diffuse light.

Colour	Calcium Concentration	Subsequent Procedure
Both tubes green	More than 50 mg./100 ml.	The urine is diluted 1 in 3
First tube green, second mauve	10–50 mg./100 ml.	The urine is used neat
Both tubes mauve	Less than 10 mg./100 ml.	A larger volume of phosphate-free supernatant is used for titration; 3 ml. is usually suitable

No failure of recovery is encountered with urines containing calcium deposits of calcium phosphate or oxalate. The urine must be shaken thoroughly before an aliquot is taken for phosphate precipitation, at which stage these calcium salts go into solution.

Bone.—Marrow and fat-free bone is prepared by refluxing with Bloor's mixture as described by Follis (1952). About 100 mg. of the prepared bone is digested at 100° C. in about 10 ml. of 3N-HCl for a few hours until solution is complete. The volume is then adjusted to 100 ml. with distilled water.

Teeth.—Whole tooth, enamel, or dentin is ground and about 60 mg. of the resulting powder is digested at 100° C. in 10 ml. of 3N-HCl, and the solution made up to 100 ml. with water.

Soft Tissues.—These usually have a calcium concentration less than 10 mg./100 ml. Homogenization with dilution lowers the calcium concentration too much for accurate analysis. Homogenization without dilution is often difficult unless special disintegrators are available, and calcium extraction has not always been complete. Hence preparation by ashing is generally advised.

About 10 g. fresh tissue is weighed. The water content can be estimated at this stage if required. The specimen is ashed overnight at about 500° C. After cooling, the ash is dissolved in a small amount of N-HCl (about 1 ml. is usually required) and the solution made up to 5 ml. with water.

Phosphate Separation from Prepared Sample

In a stoppered tube are mixed 2 ml. of the prepared sample made as above, 1 ml. tungstate reagent, and 2 ml. morpholine nitrate-nitric acid reagent. This precipitates phosphate and protein. The mixture is allowed to stand for one hour at room temperature then centrifuged. The supernatant is used for titration.

Titration

Test.—Supernatant (1 ml.) is pipetted into a small white crucible and a few drops of 40% NaOH added until the mixture is strongly alkaline (*p*H about 12). When setting up the method it is necessary to find the correct amount of alkali by taking a separate aliquot of the supernatant and bringing it up to *p*H 12 using B.D.H. 1014 indicator. With practice, this is unnecessary since the shade of the calcein-thymolphthalein indicator enables the correct *p*H to be gauged. In the latter case, calcein-thymolphthalein is added to the 1 ml. supernatant before adding alkali. Glycine buffer (5 ml.) is then added.

Blank.—This is made up of 1 ml. distilled water and 5 ml. glycine buffer. The blank titration is negligible when the water used is very pure.

Standard.—The standard consists of 1 ml. standard calcium solution (10 mg./100 ml.) and 5 ml. of glycine buffer.

A small knife point (0.5–1 mg.) of indicator is added to each solution and they are titrated with E.D.T.A. until they change colour from green to mauve which is stable for at least five seconds. At this stage adding further E.D.T.A. gives no further change of colour.

It is convenient to use a small magnetic stirrer for these titrations. If a stirrer is not available the mixture should be continually agitated during titration. This procedure is adapted from that previously described for serum calcium (Baron and Bell, 1957). The only modifications that have been made to the original titration procedure are the use of the magnetic stirrer and of opaque white dishes as the titration vessels. Daylight or a daylight bulb should be used.

Procedure for Serum.—Serum (1 ml.) is titrated directly in 5 ml. of glycine buffer; the colour change is orange-green to pink. Alternatively a blank titration can be carried out on 5 ml. of buffer solution, 1 ml. of serum added, and the titration completed: this gives the green-to-mauve end-point. Jaundice makes the end-point difficult to read but is overcome by preliminary phosphate-protein precipitation. If there is gross haemolysis, phosphate - protein precipitation should be performed, and 2 drops of 1% NaCN added to the supernatant before titration to complex the iron.

Calculation

$$\frac{\text{Test}-\text{Blank}}{\text{Standard}-\text{Blank}} \times 10 = \text{mg. calcium/100 ml. of supernatant (or untreated serum).}$$

Phosphate precipitation involves a dilution of 2 in 5, therefore,

$$\text{mg. calcium/100 ml. of prepared sample} = \frac{\text{Test}-\text{Blank}}{\text{Standard}-\text{Blank}} \times 25.$$

A further calculation may be necessary to convert the calcium concentration in a prepared sample to the calcium content of the original biological specimen.

Solutions with Low Calcium Concentration

When the prepared sample is considered to have a low calcium concentration (below 10 mg./100 ml.), 3 ml. of the supernatant is taken and 40% sodium hydroxide added until the *p*H is about 12. Because of the small amount of buffer present, great care is necessary in the addition of alkali. A final solution that is not alkaline enough will not give an end-point; a final solution that is too alkaline gives a difficult end-point. Buffer (3 ml.) and a small knife point of indicator are then added. The blank consists of 3 ml. water and 3 ml. glycine buffer.

$$\frac{\text{Test}-\text{Blank}}{\text{Standard}-\text{Blank}} \times \frac{25}{3} = \text{mg. calcium/100 ml. of prepared sample.}$$

If the calcium concentration is very low, 5 ml. supernatant and 1 ml. buffer may be used.

Micro Modification

The method can be adapted when only small specimens are available for analysis. Preparation of the specimens follows similar lines to that described

above. If ashing is necessary, about 500 mg. of tissue is weighed, ashed, and the ash dissolved in 0.1 ml. N-HCl and 0.2 ml. water.

Phosphate Separation.—Of the prepared sample, 0.2 ml. is mixed with 0.1 ml. tungstate reagent and 0.2 ml. morpholine nitrate-nitric acid reagent and centrifuged. The supernatant is taken for titration.

Titration

Test.—For the test 0.1 ml. supernatant is pipetted into a small white crucible and the *p*H adjusted to about 12 with 40% NaOH. Glycine buffer (0.5 ml.) and a very small knife point of indicator (about 0.2 mg.) are added.

Standard.—For the standard, 0.1 ml. standard calcium solution (10 mg./100 ml.), 0.5 ml. buffer, and indicator are required.

Blank.—The blank requires 0.1 ml. distilled water, 0.5 ml. buffer, and indicator.

Test standard and blank are titrated with E.D.T.A. solution diluted 1 in 5 (an E.D.T.A. solution which is more dilute than this results in a poor end-point). (For serum, use 0.1 ml.+0.5 ml. glycine buffer.)

Calculation

The calculation is as for the macro method above.

RESULTS

Each stage of the method has been examined.

Ashing

Table I shows that ashing is generally not necessary for the analysis of calcium in the biological specimens described here apart from soft tissues. Muscle (a typical soft tissue) was not accurately analysed by homogenization without ashing, and in such cases it is necessary to ash.

Table II shows that the ashing procedure adopted did not result in loss of calcium, and that, although adding acid before ashing, as described by Horner (1955), is unnecessary, dilute acid must be used to extract the ash.

Phosphate Separation Compared with Oxalate Precipitation

Serum calcium may be estimated directly without phosphate precipitation (Baron and Bell, 1957), but it is impossible to obtain a clear end-point with urine and most other biological specimens by this method because of the interference caused by phosphates (Collier, 1955).

Morpholine nitrate and tungstate (Horner, 1955) have proved entirely satisfactory for removing phosphate before titration of calcium, resulting in an easily read end-point, no loss of calcium, or introduction of interfering ions.

TABLE I

EFFECT OF ASHING ON DETERMINATION OF CALCIUM IN BIOLOGICAL SPECIMENS

Biological Specimens	Calcium Found		
	Ashed	Unashed	Units
Food I (chicken, bread, milk, fruit, and vegetable)	115 116	116 120	mg./specimen
Food II (beef, egg, margarine, and cereals)	440 440 425	422 416 432	
Faeces I	474 482	489 480	mg./24 hours
II	519 506	512 519	
III	867	861 866	
Urine I	5·4	5·4 5·6	mg./100 ml.
II	6·2 6·1	6·2 6·2	
Milk I	108	111	mg./100 ml.
II	115	121	
III	119	123	
Whole tooth	28·2	28·2	mg./100 mg.
Dentine	36·4	36·2	
Enamel	28·2	27·9	
Bone	25 0 25·0	25·0 24·9	mg./100 mg.
Muscle	5·9 5·9 5·9	4·5 6·5 5·6	mg./100 g.

The biological specimens were prepared as described in the methods section. One portion of each of the prepared samples was ashed overnight in a muffle furnace at 500°, the ash dissolved in N-HCl and diluted to volume with water. A suitable aliquot of this solution, and another portion of the sample prepared as above, were treated with tungstate and morpholine nitrate-nitric acid reagent for phosphate precipitation. Calcium was estimated on the supernatants by E.D.T.A. titration.

TABLE II

INVESTIGATION OF DIFFERENT ASHING PROCEDURES

Materials Ashed	Ash Dissolved in	Calcium (mg./100 ml.)
(a) 4 ml. serum	4 ml. water	0·5
4 ml. serum + 1 ml. conc. HCl	,,	0·4
4 ml. serum+1 ml. conc. HNO_3+1 ml. conc. HCl	0·2 ml. N-HCl 3·8 ml. water	10·0
4 ml. serum	0·2 ml. N-HCl 3·8 ml. water	10·0
(b) Serum titrated directly without ashing		10·0

(a) Specimens, each of 4 ml. of serum, were ashed overnight at 500° and the ash taken up in the mixtures shown above. Calcium was estimated by the accepted oxalate procedure of Clark and Collip (1925).
(b) The serum was also titrated directly, without phosphate precipitation, by the method of Baron and Bell (1957).

Table III shows a comparison between the results of calcium analysis by this method (which takes one and a half hours to complete) and by standard oxalate precipitation methods. It can be seen that the complexiметric method seems to give results that are slightly lower, which has also been noted for serum analysis by Wilkinson (1957).

TABLE III

COMPARISON BETWEEN RESULTS OBTAINED FOR CALCIUM ESTIMATION BY PRESENT METHOD AND A STANDARD OXALATE METHOD

Biological Specimens	Calcium Found		Units
	Present Method	Oxalate Method	
Food I	116	114	mg./specimen
	120	112	
II	422	440	
	416	400	
	432	428	
Faeces I	512	530	mg./24 hours
	519	500	
	519		
II	489	500	
	480	515	
	480	505	
Urine I	1·8	2·0	mg. 100 ml.
II	3·3	3·6	
III	5·4	5·6	
	5·6	5·6	
IV	10·0	10·4	
V	17·4	19·0	
	17·3	18·6	
VI	31·5	30·8	
VII	10·1*	10·2	
VIII	14·6*	14·6	
IX	25·5*	26	
Milk I	123	130	mg./100 ml.
II	132	140	
Whole tooth	28·2	28·6	mg./100 mg.
Enamel	36·2	36·4	
Dentine	27·9	29·0	
Bone	25·0	24·9	mg./100 mg.
	24·9		
Muscle	5·9	5·9	mg./100 g.
	5·9	5·9	

Present Method.—Specimens of food, faeces, bone, teeth, and urine were prepared without ashing and muscle with ashing as described in the methods section, phosphate was precipitated with morpholine nitrate and tungstate, and calcium determined by E.D.T.A. titration. Micro-analysis of calcium, using 0·1 ml. of the supernatant after phosphate precipitation as described in the methods section, was performed for those results marked by an asterisk.

Oxalate Method.—Specimens of food, faeces, muscle, and milk were ashed overnight at 500°, the ash dissolved in N-HCl and diluted to volume with distilled water. Bone and teeth samples were prepared as described in the methods section. Urine was used without preparation. Calcium was estimated by oxalate-permanganate procedures by the methods of King and Wootton (1956) for urine and milk and of Hawk, Oser, and Summerson (1954) for food, faeces, muscle, bone, and teeth.

This method also appears more reliable, as duplicates show closer agreement than with the oxalate methods. It must be admitted, however, that the end-point of the permanganate-oxalate titration is sharper than that found with any of the metallo-indicators for calcium which have been tried.

Precipitation of phosphate using zirconium nitrate, a method which was modified from that described by Vogel (1954), was also tried, but did not prove satisfactory. While this work was in progress, another method of phosphate precipitation using metastannate was described (Ling, 1958). Although it was usually found satisfactory it did not always give reliable results because of the need to control accurately the factors involved, in particular the concentration of nitric acid. For this reason the morpholine method (as suggested by Wilkinson, 1957) seems more suited to routine work.

Indicators

In addition to calcein-thymolphthalein the other indicators investigated were "calcon," 1-(2-hydroxy - 1 - naphthylazo)-2-naphthol-4-sulphonic acid (Hildebrand and Reilley, 1957), and "cal-red," 2 - hydroxy - 1 - (2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid (Patton and Reeder, 1956). The end-point with these indicators was considered inferior to that of the recommended "calcein-thymolphthalein," especially in the presence of serum protein. These other indicators can of course be used if the observer prefers their particular colour changes.

Titration Volumes

Provided that the *p*H was controlled, the use of a larger volume of supernatant in relation to the volume of buffer, when solutions of low calcium content are analysed, did not lead to loss of accuracy (Table IV).

TABLE IV

EFFECT OF VARYING AMOUNT OF SUPERNATANT ON RESULTS OF CALCIUM TITRATION

Urine	Supernatant (ml.)	Buffer (ml.)	Calcium (mg./100 ml.)
I	5	1	4·8
	3	3	4·9
	2	4	4·7
II	5	1	3·6
	3	3	3·6
	2	4	3·5

Twelve millilitres of each of two different urines were treated with 6 ml. tungstate reagent and 12 ml. of morpholine nitrate-nitric acid reagent to precipitate phosphate, and varying amounts of the supernatant and buffer were taken for calcium estimation by E.D.T.A. titration.

Recovery and Accuracy

Recovery experiments were carried out using calcium oleate for faeces, calcium phosphate for food, calcium chloride for urine, and calcium lactate for milk. These compounds were used because they are the commonly occurring compounds of calcium in these biological specimens. Table V shows that satisfactory recovery of calcium was obtained in all cases.

In order to test the reproducibility of the method, calcium was estimated eight times on one urine. The coefficient of variation was 3.3%, the mean calcium concentration being 17.4 mg./100 ml.

TABLE V

RECOVERY EXPERIMENTS

Biological Specimen	Calcium Found	Calcium Calculated	Units	Recovery (%)
Urine ..	11·63	(Original sample)	mg./100 ml.	
	13·6	13·6		100
	17·3	17·0		105
	21·98	21·6		104
	23·7	24·1		97
Food ..	17·6	(Original sample)	mg./100 ml. preparation	
	22·4	22·48		98
	28·9	28·89		100
Milk ..	123	(Original sample)	mg./100 ml.	
	175	173		104
Faeces ..	16·2	(Original sample)	mg./100 ml.	
	28·5	29·5		92

Calcium lactate was added to milk, calcium chloride to urine, calcium phosphate to food, and calcium oleate to faeces. The biological specimens were prepared as described in the methods section, phosphate precipitated with morpholine nitrate and tungstate and the calcium estimated by titration with E.D.T.A.

TABLE VI

EFFECT OF BARIUM AND MAGNESIUM SALTS ON ESTIMATION OF CALC

Final concentration of added magnesium (mg./100 ml.) ..	0	25
Calcium:		
mg./100 ml. bone solution ..	24·9	24·9
,, urine	3·7	3·7
Final concentration of added barium (g./24 hr. faeces) ..	0	29·4
Calcium:		
mg./24 hr. faeces	519	575
	519	580

Magnesium as magnesium sulphate was added to bone solution and urine, and barium as barium sulphate to faeces, to give the final concentrations shown below. Phosphate and protein were precipitated with morpholine nitrate and tungstate and calcium estimated by E.D.T.A. titration.

Interference

The effects of magnesium and barium on the estimation of calcium by this method were tested (Table VI).

Magnesium sulphate was added to a solution of bone (prepared as described above), and to urine, to give in each case an increase in the magnesium concentration of about 25 mg./100 ml. Although the added magnesium concentration of the urine was seven times as great as the calcium concentration no effect was observed on the calcium estimated. Similarly, added magnesium did not alter the estimated calcium concentration in bone.

A large quantity of barium, as barium sulphate (1 g. to 50 ml. prepared faeces sample), had only a small effect on the estimated calcium. This concentration of barium might be found in the faeces of a patient after barium sulphate had been given for radiological examination.

DISCUSSION

It was necessary to devise a method of calcium estimation that was applicable to any sort of biological specimen, whether of high or low calcium concentration. The method had to be accurate and speedy, and capable of performance in any biochemical laboratory without specialized equipment.

The classical method of calcium estimation in biological samples involved ashing of the sample and precipitation of the calcium as oxalate before eventual permanganate titration or gravimetric analysis (Hawk *et al.*, 1954). This method has many disadvantages. The calcium oxalate precipitate is difficult to handle when only small quantities of calcium are present, and the washing procedure is tedious if sufficient care is taken neither to leave excess oxalate nor to lose any precipitate. It is also advisable to allow eight hours to ensure complete precipitation.

In recent years methods for the final estimation of calcium in the prepared sample have been introduced utilizing either flame photometry or titration with E.D.T.A.

Flame photometry of calcium is difficult because of the weakness of the calcium bands in relation to the sodium line, and because of phosphate interference. If an ordinary commercial flame photometer is used, calcium can only be estimated after separation as oxalate (Butterworth, 1957), which is of little advantage even though washing the precipitate is unnecessary. Otherwise it is necessary to use a specially designed instrument (MacIntyre, 1957) or an expensive commercial instrument (Jackson and Irwin, 1957) which are not available in small laboratories. With these methods biological specimens have first to be prepared to eliminate the interference caused by phosphate and protein.

Titrimetric estimation of calcium with E.D.T.A. using the earlier indicators such as ammonium purpurate (Horner, 1955; Wilkinson, 1957; Dunstone, 1957) is not completely satisfactory as a routine method because the indefinite end-point cannot be determined visually. This makes it necessary to perform the titration in an absorptiometer.

The newer indicator, "calcein" (Diehl and Ellingboe, 1956), gives a sharp end-point for calcium at a *p*H of about 12.5 and magnesium is not estimated. This end-point is detectable by the naked eye without any difficulty when the fluorescence of the "calcein" is masked by thymolphthalein (Tucker, 1957). The calcium may first be separated by oxalate precipitation with its known

disadvantages: this was done by Gilbert and McGann (1958), using "cal-red" as the indicator, for the estimation of calcium in muscle. Yarbro and Golby (1958) used "calcon" as the indicator for the estimation of calcium in urine; they titrated with E.G.T.A. (ethylene glycol bis (β-aminoethyl ether) N_1N', tetra-acetic acid), removed protein if present, and claim that phosphate separation is then unnecessary. We consider that a method of calcium estimation which is generally applicable to biological materials must involve separation of calcium from phosphate, and any method which requires precipitation of calcium as oxalate is not as satisfactory in routine use as one which involves precipitation of the phosphate.

SUMMARY

A method is described for titrimetric estimation of calcium in biological specimens which does not involve oxalate precipitation. It is simple and takes about one and a half hours.

Preliminary ashing is only required for tissues of low calcium concentration.

Interference by phosphate is removed by precipitation with morpholine nitrate and tungstate. The calcium in the phosphate-free supernatant is titrated with E.D.T.A. (sodium edetate) at *p*H 12.6 using a calcein-thymolphthalein indicator. Magnesium causes no interference.

Recoveries of calcium added to food, faeces, milk, and urine were satisfactory.

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ADDENDUM

Since submitting this paper, we have tested acid alizarin black (Belcher, 1958), kindly supplied by Dr. Belcher and obtainable commercially. This indicator is stable for a few days in 0.5% aqueous solution; one drop is used for the titration. There is a sharp colour change from purple to a stable turquoise blue at the end-point, and this indicator is recommended. However, if removal of supernatant after phosphate separation with morpholine-tungstate is not done carefully, a faint blue colour may occur after adding alkali, which makes this blue end-point less easy to see.

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AN IMPROVED FLAME-PHOTOMETRIC METHOD FOR DETERMINING CALCIUM IN SERUM

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Powell (1953) introduced a method for the estimation of serum calcium using the EEL flame photometer, and claimed that it gave results in good agreement with a standard titrimetric procedure.

In this laboratory, both these methods were found to give low results when tested against standard sera, prepared by adding known amounts of calcium to serum completely decalcified by treatment with a cation exchange resin. The cause of these low results has been established, and modified procedures for avoiding them have been developed.

Materials

Resin.—Amberlite XE-66B (Charles Lennig & Co., Ltd., London) was used in the sodium form. The resin (50 g.) was prepared by treatment with 1 litre volumes of (1) 6N HCl, (2) water (twice), (3) 2N NaOH, and (4) water (twice), this sequence being repeated until the supernatant from the HCl treatment was colourless. After the next NaOH treatment, the resin was washed until the washings were neutral and chloride-free, and then air dried.

Sera of Known Calcium Content (Standard Sera).—Pooled human serum (100 ml.) was decalcified by shaking with three successive batches of "amberlite XE-66B" (sodium form, 4 g.). Sera of known calcium content (8–16 mg./100 ml.) were prepared by evaporating to dryness aliquots of a standard solution of calcium chloride, and re-dissolving the residue in suitable volumes of decalcified serum. The standard calcium solution (10 mg. Ca/100 ml.) was prepared by dissolving 0.250 g. anhydrous calcium carbonate (AR, dried at 100–110° C.) in the minimum amount of concentrated HCl, and diluting to 1 litre with water.

All analyses were carried out in duplicate. The titrimetric and flame photometric methods of Powell (1953) were used; the only modification was that precipitation of calcium oxalate was allowed to proceed overnight. Although Powell's titrimetric procedure is stated to be that of Clark and Collip (1925), it is, from his description, obviously that of Tisdall (1923).

Improved Flame Photometric Method

Reagents.—Water which had been passed through an "elgastat" deionizer was used for all reagents, standards, and dilutions.

Trichloroacetic Acid Solution.—10% w/v.

Oxalate Precipitant.—Oxalic acid, 0.63 g., 13.50 g. ammonium oxalate, and 50 g. ammonium acetate (all AR) are dissolved to 1 litre with water.

Triton X-100 (Charles Lennig & Co., Ltd., London).—2% v/v.

Solvent for Oxalate Precipitant.—Perchloric acid (AR, 72%) 0.71 ml., and 10 ml. "triton X-100" solution are diluted to 100 ml. with water.

Blank Solution.—Equal volumes of solvent and absolute ethanol.

Stock Calcium Standard.—Calcium carbonate (AR, dried at 100–110° C.), 0.250 g., and 7.1 ml. perchloric acid (AR, 72%) are made up to 1 litre with water.

Working Calcium Standard.—Stock standard, 10 ml., and 4 ml. "triton X-100" solution are made up to 40 ml. with water and mixed with 40 ml. absolute ethanol. This solution is freshly prepared for each batch of determinations and allowed to cool to room temperature before use.

Procedure for Serum.—Trichloroacetic acid (4 ml.) is added to 2 ml. serum and 4 ml. water in a centrifuge tube, mixed, and, after standing 20 min., centrifuged at 2,000 r.p.m. for 20 min. Duplicate aliquots (2.5 ml.) of clear supernatant are transferred to centrifuge tubes (15 ml. capacity) and mixed with 4 ml. oxalate precipitant and 0.6 ml. "triton X-100." After standing overnight, each tube is again centrifuged as above, and the supernatant decanted. The tube is drained by standing inverted for 1 min. on Whatman No. 42 filter paper and the mouth of the tube dried with the same paper. The precipitate is dissolved with agitation in 2 ml. solvent and the solution is mixed with 2 ml. ethanol. This solution is then sprayed in the flame photometer, set to zero with the blank solution and the sensitivity adjusted so that the working standard reads 50. The volume of the solution is sufficient to permit two or three readings to be taken if required.

Under these conditions

$$\text{Serum calcium} = \frac{\text{Test reading}}{\text{Standard reading}} \times 10 \text{ mg./100 ml.}$$

Procedure for Urine, Faeces, and Food.—The method described above is used without modification for the estimation of calcium in urine. The sample volume (usually 2 ml.) may be altered in cases where excretion is abnormal.

Faeces, or food, is homogenized in water and the homogenate weighed. Aliquots, weighed into silica crucibles, are dried in a vacuum oven, and ashed overnight at 550° C. The ash is dissolved in 6N-HCl and made up to a suitable volume with water. Calcium in aliquots of this solution is estimated as in the trichloroacetic acid extracts described above.

Results and Discussion

Accuracy of Standard Sera.—The calcium concentration of a standard serum will be correct only if the serum is completely decalcified before the addition is made. Stefanini (1948) and Hussey, Quick, Stefanini, Consolazio, and Sargent (1950) found that serum could be completely decalcified with the cation exchange resin " amberlite IR-100." Amberlite XE-66B, a finely divided form of a resin of greater exchange capacity, should act similarly.

The efficiency of the decalcification procedure was tested in various ways. No titratable oxalate precipitate could be detected in serum treated as described above by the Tisdall (1923) method. This finding merely sets an upper limit of about 0.5 mg./100 ml. for the residual calcium (Sendroy, 1944).

Samples of decalcified serum and of a standard solution of calcium containing 0.05 mg./100 ml. were ashed. The ash was dissolved in one tenth of the original volume and estimated for calcium. Whereas the recovery of calcium in the latter was complete, the former again gave no titratable oxalate precipitate, showing that the calcium concentration in the treated serum was less than 0.05 mg./100 ml.

Flame photometric estimations of standard calcium solutions with and without the addition of protein-free extract from treated serum showed that the latter contained less than 0.1 mg. Ca/100 ml.

Additions of calcium equivalent to 10 mg./100 ml. were made to samples of serum removed after each of five consecutive treatments with resin.

TABLE I

DECALCIFICATION OF SERUM BY SUCCESSIVE RESIN TREATMENTS

No. of treatments before sampling	0	1	2	3	4	5
Calcium content (mg./100 ml.) . .	19	11	10·1	10·1	10·1	10·1

The equivalent of 10 mg. calcium/100 ml. was added to each sample before estimation.

The results of analyses by the new method (Table I) show that removal of calcium from the serum is virtually complete after only two treatments with resin. Table II shows that protein and phosphate are only slightly altered, magnesium and potassium are almost completely removed, and serum sodium is increased, by three treatments with resin. Data provided by Sendroy (1944) and Smith, Craig, Bird, Boyle, Iseri, Jacobson, and

TABLE II

EFFECT OF RESIN TREATMENT ON SERUM CHEMISTRY

Constituent	Concentration	
	Before Resin Treatment	After Resin Treatment
* Protein (g.%)	5·6	5·75
Magnesium (mg.%)	3·4	0·0
Sodium (mEq./l.)	132	159
Potassium (mEq./l.)	3·7	0·1
Inorganic phosphorus (mg.%) . .	3·6	3·7

* There was no detectable difference in the electrophoretic pattern of untreated and treated sera.

Myers (1950), concerning the precipitation of calcium oxalate from serum, and by Llaurado (1954) on the amounts of sodium and potassium needed to produce detectable interference with the calcium flame, show that the changes observed in the concentrations of sodium and potassium could not have interfered with any subsequent procedure to which the standard sera were subjected. Magnesium does not interfere with flame-photometric serum calcium estimations (MacIntyre, 1957 ; Toribara, Dewey, and Warner, 1957), but it seemed possible that the removal of this element might make the serum unreliable as a standard for titrimetric procedures, since Smith *et al.* (1950) and MacIntyre (1957) have shown that some magnesium may be co-precipitated with calcium oxalate. However, the recovery of calcium in estimations on standard sera, using the Tisdall (1923) method, was unaltered by the addition of magnesium (3 mg./100 ml.) to the sera.

Flame Photometric Procedure of Powell (1953).—The results in Table III (column 1a) show that this method gave low results with standard sera. Since Sendroy (1944) demonstrated complete precipitation of calcium oxalate under comparable conditions, contamination of the precipitate by a substance which depressed the calcium flame intensity was suspected.

Washing such precipitates improved the recovery of calcium, and the results after ashing were very close to the expected values (Table III, columns 1b and 1c). This suggested that the contaminant was partially soluble.

TABLE III
ESTIMATION OF CALCIUM IN STANDARD SERA BY VARIOUS METHODS

Calcium Content of Standard Serum (mg./100 ml.)	Results (mg./100 ml.)						
	Method I			Method II		Method III	
	(a)	(b)	(c)	(a)	(b)	(a)	(b)
8·0	7·3	7·6	7·8	7·5	8·3	—	7·8
	7·0	—	—	—	8·0	—	8·2
	7·1	—	—	—	—	—	—
	7·3	—	—	—	—	—	—
	7·1	—	—	—	—	—	—
10·0	8·7	9·0	9·8	9·0	9·9	9·0	10·2
	9·2	—	—	9·2	10·1	—	10·4
	8·7	—	—	9·1	9·9	—	10·2
	8·4	—	—	9·2	10·1	—	9·9
	8·7	—	—	9·4	10·2	—	10·2
	—	—	—	—	10·0	—	—
12·0	10·4	11·1	11·8	11·5	12·2	11·0	12·0
	11·2	—	—	—	—	—	12·1
	10·3	—	—	—	—	—	12·0
	11·2	—	—	—	—	—	11·9
	10·4	—	—	—	—	—	12·2
	11·2	—	—	—	—	—	—
14·0	13·2	—	—	—	14·1	12 8	14·0
	12·0	—	—	—	—	—	13·9
	—	—	—	—	—	—	14·0
	—	—	—	—	—	—	13·9
	—	—	—	—	—	—	14·2
16·0	14·4	—	—	—	16·2	14·2	16·3
	13·5	—	—	—	—	—	15·7
	—	—	—	—	—	—	16·3
	—	—	—	—	—	—	15·9
	—	—	—	—	—	—	16·1

Methods: (I) Flame photometric (Powell). Precipitate (a) unwashed, (b) once washed, (c) ashed. (II) Titimetric (Tisdall). Precipitate washed with (a) dilute ammonia and (b) dilute ammonia saturated with calcium oxalate. (III) Improved flame photometric. (a) Without and (b) with addition of "triton X-100" to precipitant mixture.

Powell (1953) showed that the use of 0.05N perchloric acid overcame depression of calcium flame intensity due to oxalate. This observation has been confirmed by experiments in which ammonium oxalate was added to a standard solution of calcium in amounts in excess of that likely to contaminate the calcium oxalate precipitate.

Similar experiments in which phosphate was added excluded it as the cause of the low recovery of calcium, a conclusion already indicated by the improved results obtained after ashing oxalate precipitates from serum.

The high concentration of protein in serum makes contamination of an unwashed calcium oxalate precipitate by protein inevitable. Furthermore, the proximity of the iso-electric point of serum globulin (about *p*H 5.5) to the *p*H of the precipitation mixture makes additional contamination by precipitation possible. The unwashed precipitate of calcium oxalate obtained in Powell's method was found to contain about 1.25 mg. of protein. A similar amount of protein remained in the centrifuge tube after centrifugation and decantation of decalcified serum treated with *p*H 5 oxalate buffer. Qualitatively, this protein was in the form of a precipitate together with a trace of serum/oxalate mixture. This was confirmed by paper electrophoresis (veronal buffer, *p*H 8.6), the precipitate remaining at the site of application.

Addition of this protein-containing deposit from 2 ml. decalcified serum to 4 ml. of a standard calcium solution (≡10 mg./100 ml. serum calcium) depressed the flame photometer reading, giving an apparent calcium concentration of only 8.6 mg./100 ml., a value almost identical with that found for the 10 mg./100 ml. standard serum by Powell's method. The same effect was produced by adding serum globulin (1.25 mg.) prepared from human serum by half-saturation with ammonium sulphate, but albumin (1.25 mg. bovine albumin, Armour & Co., Ltd., London) caused no depression of calcium flame intensity.

The Tisdall (1923) Method.—Powell found good agreement between results obtained by his flame-photometric method and by the titrimetric method of Tisdall (1923). This agreement is surprising since it was shown above that the flame photometric method gave low results on sera of known calcium content. However, low results have been obtained by Clark and Collip (1925), and MacIntyre (1957) with Tisdall's method due to loss of calcium oxalate during the two washings of the precipitate with dilute ammonia solution. Calcium determinations on standard sera (Table III, column 2a) confirmed that Tisdall's method gave low results, although the recoveries were not so poor as those obtained with Powell's flame photometric method. Figures close to the expected values (Table III, column 2b) were obtained with the titrimetric procedure when dilute ammonia solution saturated with calcium oxalate (Stanford and Wheatley, 1925) was used to wash the precipitate.

These results show that Tisdall's titrimetric procedure is not valid as a standard for assessing other methods of calcium estimation.

Improved Flame Photometric Method.—The quantities of serum (2 ml.), water (4 ml.), and trichloroacetic acid (4 ml.) recommended provide sufficient protein-free supernatant for duplicate estimations of both calcium and inorganic phosphorus. Sendroy (1944), Chen and Toribara (1954), and Severinghaus and Ferrebee (1950) found that a trichloroacetic acid extract contained all the serum calcium, and Sendroy (1944) noted that filtration of precipitated protein led to positive errors due to calcium impurities in filter papers.

Ammonium acetate was added to the oxalate precipitant in order to effect precipitation of calcium oxalate from the trichloroacetic acid extract at *p*H 5 as recommended by Sendroy (1944). The reproducibility and accuracy of estimations on standard sera were improved by the inclusion of "triton X-100" in the precipitation mixture (Table III, columns 3a and 3b). The detergent was thought to prevent particles of the precipitate from sticking to the sides of the tube or floating on the surface of the mixture.

Kingsley and Schaffert (1954) showed that calcium flame intensity was increased by the addition of organic solvents to the spraying mixture. Using the EEL flame photometer, the emission of a standard calcium solution was approximately doubled by adding 0.25 volumes of acetone, and trebled by adding an equal volume of methanol or ethanol. Greater concentrations of solvent made the flame unstable.

Fig. 1 shows that a linear response was obtained over a four-fold range of calcium concentration (≡ 5–20 mg. Ca/100 ml. serum).

Results obtained with the improved method using sera of known calcium content are shown in Table III (column 3b). Readings of duplicates agreed to within 4% and were usually identical.

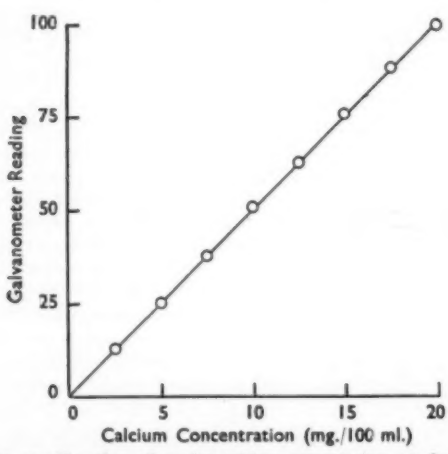

FIG. 1.—The effect of varying calcium concentrations on flame photometer readings. The standard solutions were sprayed after dilution as described for the working standard. Sensitivity was adjusted so that the 10 mg./100 ml. standard gave a reading of 50.

TABLE IV

RECOVERY OF CALCIUM ADDED TO URINE, FAECES, AND FOOD

	Calcium Added (mg.)	Calcium Found (mg.)	Recovery (%)
Urine	0·0	2·4	—
	2·0	4·4	100
	5·0	7·4	100
Faecal homogenate	0·0	6·5	—
	2·0	8·5	100
	4·0	10·3	95
	6·0	12·4	97
Food homogenate	0·0	1·5	—
	0·5	1·99	96
	1·0	2·48	98

Additions were made to samples of urine (100 ml.) or homogenate (5 g.).

The results of recovery experiments in which known amounts of calcium were added to urine or to homogenate of food or faeces are shown in Table IV.

Recoveries were excellent.

Summary

A method is described for the preparation of sera of known calcium content. It is shown, using these sera, that low results are given by Powell's (1953) method and Tisdall's (1923) method due respectively to contamination of the oxalate precipitate by protein and to washing losses. An improved method, using the EEL flame photometer, for the estimation of calcium in serum, urine, and faeces is described.

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STUDIES WITH A MORE RAPID METHOD OF VITAMIN B_{12} ASSAY UTILIZING *EUGLENA GRACILIS**

BY

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The use of microbiological assay techniques for the determination of vitamin B_{12} concentrations in serum is well established. Various micro-organisms have been used for this purpose, each with specific advantages. The use of *Euglena gracilis* has become widespread since it was first introduced by Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949). The reports of Mollin and Ross (1952), Ross (1952), Pitney and Beard (1953), Lear, Harris, Castle, and Fleming (1954), and others using this organism have established the ranges of vitamin B_{12} concentration in the serum in the normal and in various pathological states, especially in pernicious anaemia, a disease characterized by a deficiency of vitamin B_{12}.

Hutner, Bach, and Ross (1956) reported on studies with a new strain of *E. gracilis*, the *z* strain. With it, assays were conducted in a new medium after five days of incubation at 25° to 30° C., instead of the seven days required by the older methods employing the *bacillaris* strain.

This new method has been compared with the older one in order to determine its reproducibility, and whether it represents a practical improvement in the assay of vitamin B_{12} in serum.

Materials and Methods

The *z* strain of *E. gracilis*‡ was subcultured in the basal medium, as described by Hutner *et al.* (1956), with the addition of 0.2% tryptone§ and 50 $\mu\mu$g. of cyanocobalamin per ml. The medium and organism for the *bacillaris* strain assay were as described by Mollin and Ross (1952). This strain was subcultured for maintenance in the basal medium with the addition of 150 $\mu\mu$g. of cyanocobalamin per ml.

The preparation of the inoculum for both methods of assay was similar, but differed from the heavy, washed inoculum recommended by Hutner. Instead, a four-day culture was diluted 10 to 15 times with double strength medium to a density corresponding to a reading of 50 to 60 on the Klett-Summerson photoelectric colorimeter. Each culture tube was then inoculated with 0.05 ml. of this inoculum using a 1.0 ml. serological pipette.

The assay was carried out as described by Lear *et al.* (1954). Serial halving dilutions to 1/128 were made of 2 ml. of serum in double strength medium. The final volume in each culture tube was 4 ml. Similar serial dilutions were made of a stock solution of cyanocobalamin containing 200 $\mu\mu$g. of vitamin B_{12} per ml. prepared from "cyanocobalamin" U.S.P.‖ Both sets of dilutions, those of the sera to be assayed and those of the standard, were boiled for 15 minutes in a water-bath in order to free the bound vitamin. After cooling, they were inoculated with the test organisms as described above. One tube of medium was inoculated without added cyanocobalamin as a blank, control tube. The racks of culture tubes were placed on a glass shelf in an incubator illuminated from above and from below by white fluorescent lamps, and were moved about daily within the incubator in order to insure uniform lighting of all tubes. The temperature was maintained between 25° and 30° C.

The assays using the *bacillaris* strain were read after seven days of incubation, while those using the *z* strain were read after six days. The intensity of green colour in each tube was determined in a Klett-Summerson photoelectric colorimeter using a red filter (λ=640–700 mμ). The machine was adjusted to zero with the inoculated blank, control tube of each assay so as to read only the increase in colour above that of the inoculum.

With sterile, acid-washed syringes employed only for this purpose blood samples were taken from patients and allowed to clot in sterile, acid-washed, screw-capped tubes. The serum was transferred to similar sterile tubes and kept frozen until used. Except where specifically described, care was taken to exclude from the series described below any patients who had received vitamin B_{12} therapy within two or three years.

*The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School.

†Recipient of a National Research Fellowship in the Medical Sciences.

‡Obtained from the Culture Collection of Algae, Department of Botany, Indiana University.

§Obtained from Difco Laboratories, Detroit, Michigan.

‖Obtained from the Board of Trustees, U.S. Pharmacopeial Convention, Inc.

Results

The average serum values obtained by one or the other method for hospital "normals," for patients with hepatic cirrhosis, and for patients with vitamin B_{12} deficiency are compared in Table I. Equal amounts of samples of the serum of 27 of these patients were studied by both methods. The average values are shown in Table I, while the 27 individual serum values are plotted in Fig. 1. The hospital normals either had no clinical symptoms or signs of vitamin B_{12} deficiency or did not respond later to vitamin B_{12}. The patients listed as having hepatic cirrhosis had both clinical and biochemical evidence of cirrhosis of the liver. Some of the hospital normals may also have had diseases of the liver, as no attempt was made to check all patients for hepatic dysfunction. Of the four patients who seemed to have borderline vitamin B_{12} deficiency, one had a delayed and another a suboptimal reticulocyte response to vitamin B_{12} therapy. A third patient diagnosed as a case of pernicious anaemia had persistently low red cell and haemoglobin levels until given more than ordinarily adequate parenteral vitamin B_{12} therapy. The fourth, diagnosed as early pernicious anaemia, was only slightly anaemic before therapy with vitamin B_{12}. The last two patients showed only occasional abnormal red cell precursors in the bone marrow. The patients considered to have manifest vitamin B_{12} deficiency all had the blood and bone marrow changes and the response to parenteral vitamin B_{12} administration characteristic of this deficiency.

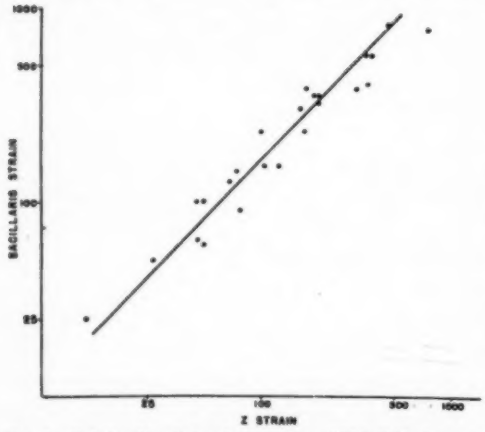

FIG. 1.—Relationship of vitamin B_{12} values of 25 individual sera as obtained by assays using two different strains of *E. gracilis*. The vertical co-ordinate of each point represents the serum level according to the *bacillaris* strain assay; the horizontal co-ordinate is the value according to the *z* strain assay in $\mu\mu$g. per ml. Two additional points (not shown) extend the apparent trend beyond the limits of the figure with co-ordinates of 1,446–1,400 and 1,600–1,235 $\mu\mu$g. per ml., respectively.

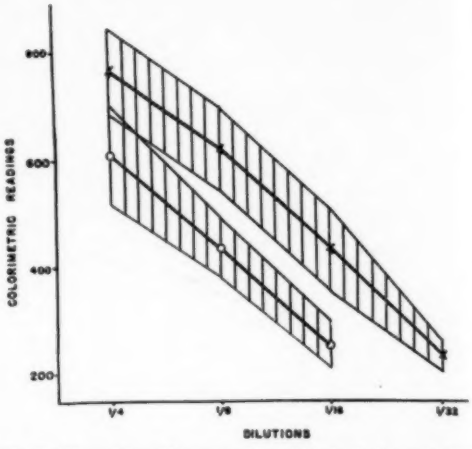

FIG. 2.—Comparison of average values of 20 standard curves from routine assays obtained with the *bacillaris* strain (O - - - O) and with the *z* strain (X - - - X) of *E. gracilis*, respectively. The cross-hatched areas represent ± 1 standard deviation. The vertical scale is for Klett–Summerson colorimeter readings; the horizontal scale indicates serial double dilutions of the vitamin B_{12} standard containing 200 $\mu\mu$g. per ml.

As shown in Table I, the *z* strain of *E. gracilis* in the new medium gave values for serum vitamin B_{12} which were consistently lower than those obtained with the *bacillaris* method. That the

TABLE I

COMPARATIVE RESULTS OF ASSAYS FOR SERUM VITAMIN B_{12} BY *BACILLARIS* AND *Z* STRAINS OF *E. GRACILIS*

Type of Patient	*Bacillaris* Assay		Both Assays			*Z* Strain Assay	
	Number of Patients	Average B_{12} Level*	Number of Patients	Average B_{12} Levels		Number of Patients	Average B_{12} Level
				Bacillaris	*Z* Strain		
Hospital "normals"	32	499±205	14	441±176	282±167	27	305±229
Hepatic cirrhosis	4	1,840±512	2	1,523±77	1,318±84	4	1,483±224
B_{12} deficiency, borderline ..	4	144±10	4	144±10	93±21	4	93±21
" " manifest ..	22	61±26	7	70±26	43±19	11	44±15

* Average serum vitamin B_{12} values in $\mu\mu$g. per ml. ± 1 standard deviation.

values obtained with the new method are proportional to those obtained with the old technique is shown in Fig. 1, in which the values obtained for equal samples of 27 sera assayed by both methods are plotted on log-log paper. A linear relationship is apparent.

The reason for this consistent difference in the results of the two methods becomes apparent when the vitamin B_{12} standard dilution curves of the assays performed by each are compared (Fig. 2). Here, the new technique gave consistently higher values than did the *bacillaris* method. However, the growth curves for aliquots of serum samples showed relatively small differences. Thus, the presence of serum seems to enrich the medium of the *bacillaris* strain technique, and so gives more growth than does a similar concentration of vitamin B_{12} without serum. This apparently explains the higher calculated serum vitamin B_{12} concentrations obtained with the *bacillaris* method.

Effect of Different Methods of Inoculation.—Hutner *et al.* (1956) recommend the use of a heavy, washed inoculum for the assay with the new medium. That washing tends to accelerate the growth of *E. gracilis* has been shown by Østergaard Kristensen (1955). Consequently, two sets of standard curves were run with the *z* strain of *E. gracilis*, using the medium recommended for this strain. One set of serial dilutions and a blank, control tube were inoculated with a dilute, unwashed culture of the organism as described under "methods." The other set and its blank control tube were inoculated as recommended by Hutner *et al.*, i.e., an eight-day culture of organisms in 15 ml. was washed in fresh culture medium and resuspended in 10 ml. of fresh medium. One drop of this suspension was added to each culture tube. Both sets of dilutions were incubated together, and each was assayed in quadruplicate. After first adjusting the colorimeter to zero with the blank tube of each series, duplicate sets of each assay were read after five days and after six days of incubation, respectively. The average density values of these duplicate dilutions are shown in Fig. 3.

The heavy, washed inoculum produced a considerable degree of colour in the blank, control tubes, reading 80–90 when compared with water. The blank tubes of the assays using the light inoculum read only 0–10 when compared with water. As shown in Fig. 3, the heavy inoculum grew sufficiently in five days to provide a usable growth curve, and showed approximately the same growth (new green colour) as did the light inoculum after six days of incubation. Results similar to those obtained after six days with the light inoculum described above would thus be expected after five days with the heavy inoculum. This was found to be the case.

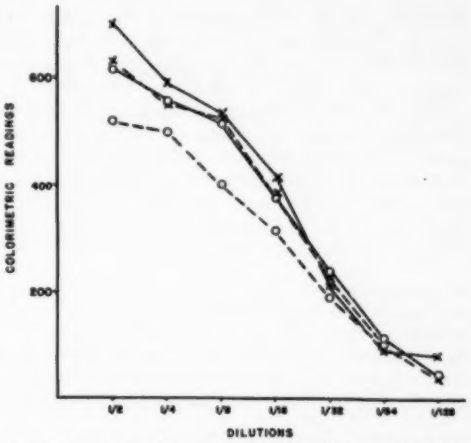

FIG. 3.—Comparison of average values of duplicate growth dilution curves of the *z* strain of *E. gracilis* using "light" (open dots) and "heavy" (crosses) inocula. The values were read after five days (dashed curves) and after six days (solid curves) of incubation, respectively. Vertical and horizontal scales are as in Fig. 2. Note that after six days the results with both light and heavy inocula are similar.

Effect of Lactic Acid.—One difficulty noted with the new assay was that the 1:4 dilutions of serum invariably coagulated during the boiling, and that the 1:8 dilutions often became cloudy during the period of growth of the organisms, giving erroneously high readings for these dilutions in the colorimeter. Dr. Hutner in a personal communication suggested that this might be due to alkalinization of the medium as a result of heavy growth. At his recommendation, lactic acid was added to the medium to a concentration of 0.1%. Although the lactic acid did not alter the assay, it failed to prevent turbidity or coagulation.

Discussion

It is evident that the new method of assay using the *z* strain of *Euglena gracilis* and a new enriched medium will allow accurate determinations of vitamin B_{12} levels in serum. It permits these determinations to be made in less time (five or six days) than is required for the assay employing the *bacillaris* strain of *E. gracilis* currently in use (seven or eight days). The range of calculated serum values obtained is consistently lower,

probably due to more growth in the absence of serum (standard curves) than was obtained with the medium of Mollin and Ross. These lower serum values tend to compress the range for vitamin B_{12} deficiency, and to make more difficult the interpretation of results in the low normal range. This objection will probably become less important as more experience is obtained with the new method; and there is clearly a theoretical objection to the non-specific, growth-stimulating effect of serum in the older assay.

Summary

Comparative assays of serum for vitamin B_{12} were carried out using the *z* strain of *Euglena gracilis* grown in a new enriched medium (Hutner), and also by the older method of Mollin and Ross (1952), employing the *bacillaris* strain. The new method gives reproducible values proportional to, but lower than, those obtained with the older method. It shortens by one or two days the growth period required.

We are indebted to Mrs. Felicita Fradera for technical assistance.

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MONILIASIS IN ACUTE LEUKAEMIA

BY

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The prolonged use of antibiotics, cortisone, or a combination of both has resulted in an increase in infections with monilia. With the recent advances in the treatment of acute leukaemia to include anti-metabolites, corticoids, and antibiotics, an increased incidence of monilial infections might be expected. This paper reports the clinical and necropsy findings in five cases of acute leukaemia with moniliasis which have occurred at this hospital during the past two years. Four of these were treated with 6-mercaptopurine (6-M.P.), corticoids, and antibiotics. The fifth was untreated in hospital, but probably received antibiotics for some time before admission.

Case Reports

Case 1.—D. P., a woman aged 59 years, a laundress, was first admitted on September 7, 1955, complaining of dyspnoea, fatigue, and sore throat. A blood count showed: haemoglobin 7.7 g. per 100 ml., white blood cells 12,000 per c.mm., platelets 26,000 per c.mm. A differential count showed 50% myeloblasts.

Procaine-penicillin, 100,000 units b.d., 6-M.P., 25 mg. five times daily, and cortisone, 50 mg. b.d., were given. Because of a falling blood count 6-M.P. was reduced to 25 mg. b.d. and terramycin, 250 mg. six-hourly, was substituted for penicillin. Bronchopneumonia developed and the antibiotic was changed to erythromycin, 2 g. daily. The patient was discharged much improved on 6-M.P., 25 mg. b.d., and prednisone, 25 mg. b.d.

She was admitted the second time on January 12, 1956, complaining of sore throat. A blood count showed: haemoglobin 10 g. %, white blood cells 2,300 per c.mm., with a normal differential count. The patient died four days after admission.

Necropsy.—There was purpura of the arms and extensive ulceration and crusting with a yellowish-white membrane on the posterior third of the tongue, epiglottic folds, pharynx, and oesophagus. There were submucosal haemorrhages in the stomach and intestines, and extensive bronchopneumonia. The red marrow occupied the entire femoral shaft. Findings in the other organs were not relevant.

Histology. — The oesophagus was extensively ulcerated with numerous mycelia on the surface and in the deeper tissues. The inflammatory exudate was largely lymphocytic and mononuclear, but occasional polymorphs were present. The lungs showed suppurative bronchopneumonia, but fungi were not detected in the lesions. The bone marrow was extensively replaced by leukaemic cells, but they had not infiltrated the liver, spleen, or kidneys. The spleen showed numerous iron-containing macrophages.

Case 2.—N. K., a woman aged 45, a housewife, was first admitted on October 28, 1955, as an emergency with prolapsed bleeding piles. A blood count showed: haemoglobin 8.9 g. per 100 ml.; white blood cells 1,900 per c.mm., platelets 36,000 per c.mm., and a differential count, showing granulopenia with 17% lymphoblasts. A marrow puncture showed 75% lymphoblasts.

Treatment was with blood transfusions, 6-M.P., 50 mg. b.d., and prednisone, 5 mg. six-hourly. A small but definite haematological improvement occurred, and the patient was discharged still receiving 6-M.P., 50 mg. b.d., and prednisone, 25 mg. b.d. She was admitted for the second time in December 7, 1955, complaining of pyrexia, diarrhoea, and sore throat.

The epiglottis and soft palate were diffusely ulcerated, and cultures produced a profuse growth of *Monilia albicans.* The blood picture showed a relapse which was treated with blood transfusions, and the dosage of 6-M.P. reduced to 25 mg. daily. One per cent. gentian violet was applied locally to the mouth, and achromycin, 1 g. daily, was given because of pyrexia. She was discharged clinically and haematologically improved. She remained well as an out-patient on 6-M.P., 25 mg. daily, and prednisone, 20 mg. daily. She was finally admitted on June 6, 1956, with a recurrence of the haemorrhoids and laryngitis, and a haematological relapse. Monilia was not isolated from repeated swabs of the mouth. Treatment was with blood transfusions, penicillin, 1 mega unit daily, tetracycline, 1 g. daily, 6-M.P., 25 mg. daily, and prednisone, 20 mg. daily, but there was no response and the patient died on July 1, 1956.

Necropsy.—There were ecchymoses on the arms and legs, and firm, yellowish, raised plaques and ulcerated areas on the posterior third of the tongue, tonsils, larynx, and oesophagus. Ulcers with raised edges and yellowish, necrotic centres were found in

the stomach, duodenum, small intestine, and, to a lesser extent, in the ascending colon. Massive haemorrhage had occurred into the wall of the caecum and the retroperitoneal tissues. The liver was enlarged and pale with numerous firm, yellowish nodules approximately 1 cm. in diameter in both lobes. The spleen was enlarged (300 g.) and brown. The lymph nodes were not enlarged. The bone marrow was red in the upper third of the shaft of the femur. There were no other relevant findings.

Histology.—The oesophageal lesions were similar to those in Case 1. The ulcers in the stomach and intestines showed necrosis in the centre with numerous fungi on the surface and in the deeper tissues. At the margins of the ulcers, areas of necrosis were present in the submucosa and these also contained large numbers of mycelia (Fig. 1). The cellular reaction consisted of lymphocytes, histiocytes, and occasional plasma cells. Many cells showed pyknosis and karyorrhexis and it is possible that some were leukaemic. The nodules in the liver consisted of extensive areas of necrosis with a cellular exudate of lymphocytes, plasma cells, and mononuclears (Fig. 2). Mycelia and yeast forms were found in large numbers. The nodules were sharply demarcated from the surrounding liver tissue and in some there was early fibrosis at the periphery. There was no evidence of leukaemic infiltration of the liver.

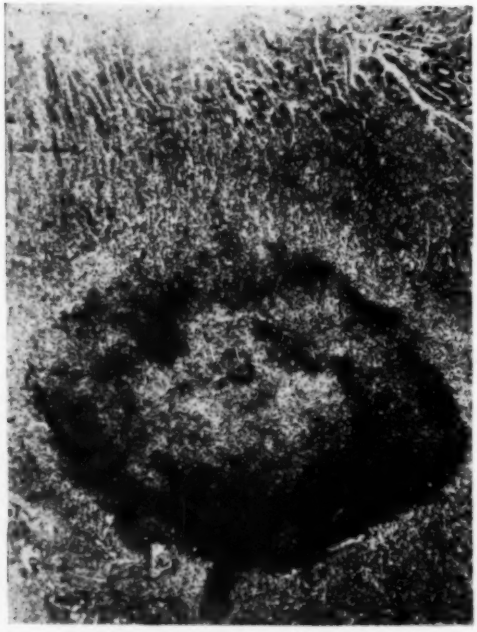

FIG. 1.—Lesion in submucosa of stomach. Masses of fungi are present. Case 2. P.A.S. × 25.

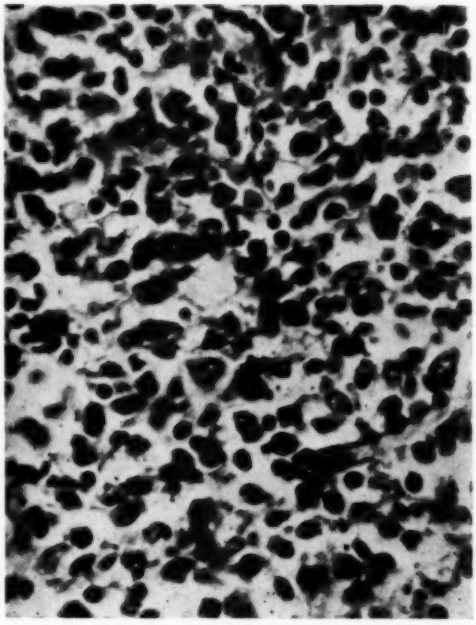

FIG. 2.—Edge of liver lesion showing cellular reaction of lymphocytes, mononuclears, and plasma cells. Case 2. Haematoxylin and eosin × 560.

Cells resembling leukaemic cells were found throughout the pulp of the spleen, but the architectural pattern was preserved. Numerous iron-containing macrophages were present. The bone marrow was extensively replaced by leukaemic cells. Other organs showed no relevant features.

Case 3.—C. L., a man aged 22, a machinist, was first admitted on March 5, 1955, complaining of dyspnoea and a haemorrhagic rash for three weeks. His temperature was 101° F. A blood count showed: haemoglobin 4.1 g. per 100 ml., white blood cells 10,100 per c.mm., platelets 23,000 per c.mm., a differential count showing 36% lymphoblasts.

Treatment was with blood transfusions, crystalline penicillin, 1 mega unit six-hourly, 6-M.P., 25 mg. five times daily, and cortisone, 25 mg. six-hourly. There was some haematological improvement, and the patient was discharged on cortisone and 6-M.P.

The patient was admitted for the second time on May 2, 1955, with an abscess of the groin, which was treated with achromycin, 250 mg. six-hourly, cortisone, 25 mg. six-hourly, and 6-M.P., 25 mg. five times a day. The dose of 6-M.P. was reduced to 50 mg. daily because of granulopenia. He was discharged clinically and haematologically improved, and continued on 6-M.P. and cortisone as an out-patient and had a complete haematological remission.

He was admitted for the third time on January 30, 1956, with an indolent leg ulcer, still in a haematological remission. He was continued on 6-M.P. and cortisone. The patient was admitted for the fourth time on May 11, 1956, with a cervical abscess from a septic tooth. He was treated with achromycin, 250 mg. six-hourly. The abscess slowly subsided, but the blood picture showed a relapse. He was discharged on cortisone and 6-M.P. He was finally admitted on August 9, 1956, acutely ill. He had pains in the chest, the liver was enlarged and tender, and the blood picture showed a relapse. Treatment was continued, but he deteriorated and died on September 4.

Necropsy.—There was diffuse brown pigmentation of the skin and multiple petechial haemorrhages. The fauces, uvula, epiglottis, and upper half of the oesophagus showed extensive ulceration and a yellowish white membrane on the surface. The stomach contained four small ulcers with raised edges. The intestines were normal. The liver was grossly enlarged and contained multiple yellowish nodules, 1–2 cm. in diameter. The right lobe contained a large yellowish mass, 12 cm. in diameter. The spleen showed yellowish nodules, 2–3 cm. in diameter. Similar rounded nodules, 1 cm. in diameter, were found in the lungs, especially at the periphery. The upper two-thirds of the femur contained reddish marrow. There were no other relevant findings.

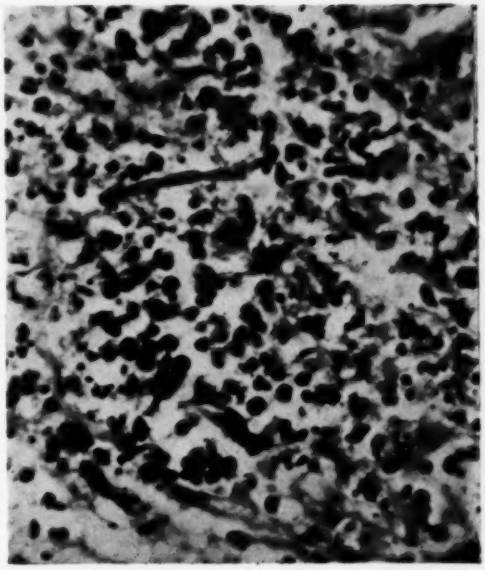

FIG. 4.—Extensive pyknosis and karyorrhexis in a liver nodule. Occasional mycelia and yeast forms are present. Case 3. Haematoxylin and eosin × 560.

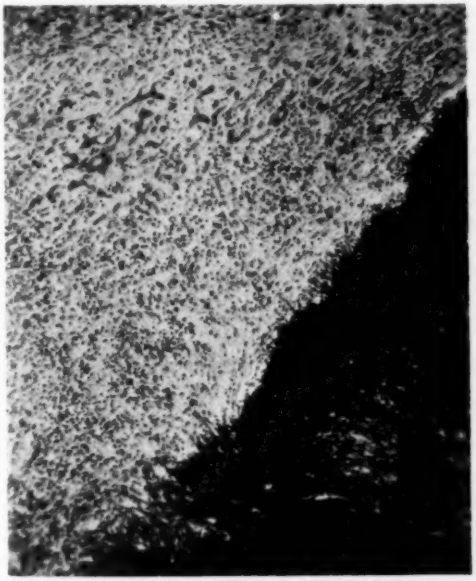

FIG. 3.—Liver nodule with masses of fungi and a peripheral cellular reaction. Case 3. P.A.S. × 75.

FIG. 5.—Nodule in spleen with extensive necrosis. A heavy deposit of iron pigment is seen in the surrounding tissue. Case 3. Haematoxylin and eosin × 25.

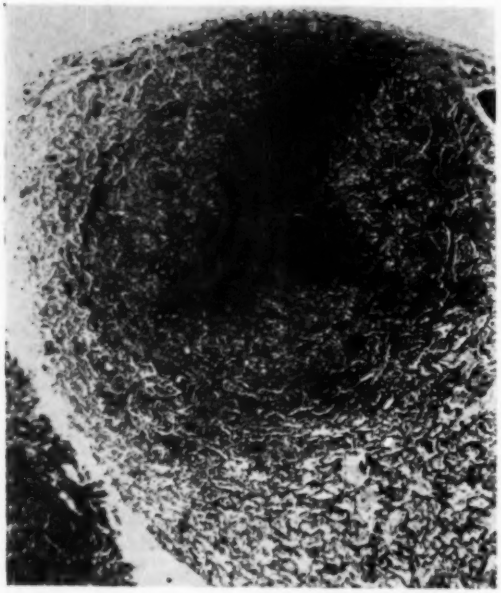

FIG. 6.—Sharply demarcated nodule in lung with central necrosis. Case 3. Haematoxylin and eosin × 25.

Histology.—The lesions in the oesophagus and stomach were similar to those in Case 2. The liver nodules showed zones of necrosis and a cellular exudate of mononuclears and lymphocytes. Numerous mycelia and yeast forms were found in the necrotic areas and between the cells (Fig. 3). As in Case 2 there was extensive pyknosis and karyorrhexis (Fig. 4). The large nodule in the right lobe showed similar appearances to the smaller nodules, but there was much more cellular reaction. There was no evidence of leukaemic infiltration in other parts of the liver. The nodules of the spleen showed a structure similar to those in the liver (Fig. 5). Numerous iron-containing phagocytes were present, but there was no evidence of leukaemic infiltration. The nodules in the lungs appeared similar to those in liver and spleen, but there was more cellular necrosis (Fig. 6). Numerous mycelia and yeast forms were present. A cervical lymph node showed necrosis, and fungi could be demonstrated in small numbers. The marrow was extremely hypoplastic and contained numerous iron-containing phagocytes.

Case 4.—J. W., a woman aged 57, a housewife, was first admitted on April 15, 1955, complaining of dyspnoea, fatigue, and pain in the chest for two days. The temperature was 102° F., and she had a left lower lobe pneumonia. A blood count showed: haemoglobin 9.5 g. per 100 ml., white blood cells 5,300 per c.mm., and a differential count showed 1.5% myeloblasts. She was treated with penicillin, 1 mega unit, and terramycin, 2 g. daily, for one week. A tentative diagnosis of diffuse carcinoma was made because of the blood picture. The patient improved and was discharged.

She was admitted finally on September 1, 1956, complaining of lassitude and mouth ulcers. A blood count gave: haemoglobin 4.4 g. per 100 ml., white blood cells 550 per c.mm., platelets 10,000 per c.mm.; blood films showed 1% pre-myelocytes. Sternal marrow showed 38% myeloblasts.

She was treated with blood transfusions, oxytetracycline, 1 g., cortisone, 100 mg., and 6-M.P., 50 mg. daily. A throat swab on September 29 showed a moderate growth of *Monilia albicans*, which was treated locally with 1% gentian violet and "nystatin," one tablet (500,000 units) four times a day. The patient died two days later.

Necropsy.—The tonsils, epiglottis, and upper two-thirds of the oesophagus were ulcerated and covered with a firm, yellowish membrane. The stomach and colon were normal. Three ulcers with long axis transverse were found in the small intestine. One small subcapsular nodule was present in the liver. The lungs showed an extensive bronchopneumonia. The hilar and cervical lymph nodes were enlarged and necrotic. The upper two-thirds of the femur contained reddish-brown marrow. There were no other relevant findings.

Histology.—The lesions in the oesophagus and small intestine were similar to those in Cases 2 and 3, and many mycelia were present in the lesions. The subcapsular nodule of the liver was entirely necrotic. Fungi were not demonstrated. There was no evidence of leukaemic infiltration. Extensive suppurative bronchopneumonia with no demonstrable fungi was found. Extensive necrosis was found in the hilar and cervical lymph nodes, but fungi were not demonstrated. Numerous iron-containing phagocytes were in the spleen, but no evidence of leukaemic infiltration was found. The bone marrow was hypoplastic.

Case 5.—H. S., a woman aged 64, a housewife, was admitted on May 26, 1957, complaining of anorexia, loss of weight, and sore throat. She had been treated at home for pneumonia, but the treatment was not known. She had extensive moniliasis of the tongue and palate. A blood count showed: haemoglobin 6.4 g. per 100 ml., white blood cells 600 per c.mm. A differential count showed 20% monoblasts. The sternal marrow was hypercellular with 65% of cells of the monocytic series. The patient died four days after admission. No specific treatment was given.

Necropsy.—A few small areas of pale, white crusting were found on the anterior surface of the tongue, soft palate, and uvula. The oesophagus was extensively involved by similar lesions. The stomach and small intestine were normal, but extensive

superficial ulceration was present in the rectum. The liver was pale but not enlarged. The spleen was normal. The cervical and para-aortic lymph nodes were enlarged. The marrow was pale brown in the upper half of the shaft of the femur. There were no other relevant findings.

Histology.—The oesophageal and rectal lesions appeared similar to those described in Cases 2 and 3. The liver, spleen, and kidneys showed no evidence of leukaemic infiltration. Reactive hyperplasia was present in the lymph nodes and the bone marrow was extensively replaced by leukaemic cells.

Discussion

Histology of the Monilial Lesions.—Nearly all the lesions showed necrosis, and the liver nodules in Case 2 were the only examples where fibrosis was noted at the periphery. The extent of the cellular reaction varied from case to case, but was predominantly lymphocytic and mononuclear with variable numbers of plasma cells. In Case 2 there is a possibility that some of the cells were leukaemic. Giant cells and foam cells as described by Ruhrmann and Adam (1955) were not found. Mycelia and yeast forms were demonstrated in large numbers within the lesions. The organisms were readily detected in routine haematoxylin and eosin preparations, but were more easily found with the periodic-acid-Schiff reagent. In all cases except Case 1 the fungi were identified by culture either during life or post mortem. In the gastro-intestinal tract the fungi were sometimes confined to the surface but often were found deeply in the tissues. Torack (1957) observed in his cases that with antibiotics alone the fungi were frequently confined to the surface in gastro-intestinal lesions, whereas in the patients receiving corticoids and antibiotics they had often penetrated to the deeper tissues. The possibility of secondary infection of leukaemic deposits was considered, but, as all lesions examined had large numbers of monilia and no uninvolved leukaemic deposits were detected, this appears to be unlikely.

Relation of Moniliasis to Treatment.—These five cases are the only examples of moniliasis in the necropsy records of the General Hospital, Birmingham, for the last 10 years. During this period necropsies have been performed on 69 cases of leukaemia, 51 of which were considered to be acute. Twelve of these cases had been treated with 6-M.P., corticoids, and antibiotics for varying periods and four showed moniliasis at necropsy. Moniliasis was encountered only once (Case 5) in the other cases, either having no specific treatment, antibiotics alone, or cortisone and antibiotics. Moniliasis was not encountered in a series of 55 necropsies on patients treated with prolonged cortisone therapy for various diseases, and half of these patients were also receiving antibiotics (Dr. K. R. Thornton, 1958, personal communication).

Since the introduction of antibiotics there has been an increased number of reported cases of gastro-intestinal and visceral involvement with monilia (Taylor and Rundle, 1952; Brown, Propp, Guest, Beebe, and Early, 1953; Schaberg, Hildes, and Wilt, 1955; Matthias and Rees, 1956). Occasional reports have appeared of moniliasis developing during cortisone therapy (Soffer, Levitt, and Baehr, 1950; Levy and Cohen, 1955), or after cortisone and antibiotics (Torack, 1957). A few cases of generalized moniliasis have been reported in leukaemias (Stenderup, Bichel, and Kissmeyer-Nielsen, 1956; Torack, 1957), and Zimmerman (1950) refers to seven cases of generalized moniliasis seen by Dr. Farber in children. Although full details of these seven cases have not apparently been published, five were of acute leukaemia treated with folic acid antagonists and antibiotics.

In view of the multiple therapeutic agents employed in four of the present cases and the debilitating type of disease, it is not possible to incriminate any particular group of drugs. Experimental evidence of an enhancing effect on the pathogenicity of monilia in experimental animals has been produced by Seligmann (1952) and Winter and Foley (1956). An enhancing effect of cortisone has been shown by Syverton *et al.* (1952), Seligmann (1953), and Winter and Foley (1956), but Winner (1958) believes that cortisone has no such effect. We have been unable to find experimental evidence on the effect of 6-mercaptopurine on monilia infections, but the high incidence of moniliasis in patients treated with 6-M.P., cortisone, and antibiotics suggests that 6-M.P. may further increase the risk of disseminated fungus infections.

Conclusion

Antibiotics, particularly of the broad range type, e.g., tetracyclines, should be used for limited periods only in acute leukaemia, and antifungal agents such as "nystatin" may be of value in preventing gastro-intestinal and generalized moniliasis.

We wish to thank the physicians of the General Hospital, Birmingham, for access to the clinical records.

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THE SENSITIVITY OF STAPHYLOCOCCI AND OTHER WOUND BACTERIA TO ERYTHROMYCIN, OLEANDOMYCIN, AND SPIRAMYCIN

BY

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Sensitive strains of *Staphylococcus aureus* can acquire resistance to all three of the antibiotics erythromycin, oleandomycin, and spiramycin when grown in culture media containing subinhibitory concentrations of any one of them (Jones, Nichols, and Finland, 1956). In contrast with the uniform pattern of resistance to the tetracyclines, however, staphylococci isolated from patients are often described as resistant to one antibiotic of the erythromycin group and sensitive to another (Hudson, Yoshihara, and Kirby, 1956; Ross, 1956; Needham and Geraci, 1956). Garrod (1957) used the term "dissociated resistance" to describe the state of organisms resistant to erythromycin but sensitive to oleandomycin or to spiramycin; he has shown that cultures of such strains include a minority of erythromycin-resistant organisms, and these grow selectively in the presence of erythromycin.

Following the increased therapeutic use of erythromycin, many burns in this Unit carry erythromycin-resistant staphylococci (Lowbury, 1957). In routine sensitivity tests by a ditch plate method, erythromycin-resistant strains were sometimes resistant to oleandomycin, but more often sensitive or moderately sensitive to that antibiotic; on continued incubation of the test cultures for a further 24 hours, however, strains showing this dissociated resistance to erythromycin usually appeared to have become resistant also to oleandomycin. Such findings were supported by tube dilution tests.

In this paper data are given on the incidence of dissociated and cross resistance of *Staph. aureus* from burns, and experiments are described which throw light on the apparently transient oleandomycin sensitivity of erythromycin-resistant strains. Some notes also are included on the sensitivity and resistance of *Streptococcus pyogenes* and *Clostridium welchii* to these antibiotics.

SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* FROM BURNS TO THE ERYTHROMYCIN ANTIBIOTICS

Materials and Methods

Strains of coagulase-producing staphylococci (*Staph. aureus*) were isolated from burns of in-patients and examined by methods most of which are described elsewhere (Lowbury, Topley, and Hood, 1952).

Since February, 1957, all strains of *Staph. aureus* have been tested for sensitivity to erythromycin and, for separate periods, to oleandomycin, to spiramycin, and to both of these antibiotics. A ditch plate method was used, parallel ditches being cut from opposite sides of a horse blood agar plate and filled with nutrient agar containing 10 μg./ml. of the antibiotics (except spiramycin, which in the later months of the study was included at a concentration of 50 μg. per ml.). Small loopfuls of undiluted overnight broth culture were inoculated on this medium, the loop being carried from the middle of the plate first across one ditch and then across the other. A sensitive and a resistant control strain were inoculated on every plate. The plates were incubated at 37° C. and examined with the naked eye after 18 hours' and again after 42 hours' incubation. The manner of reading and recording is described under Results and in the footnote to Table I.

Staphylococci were tested by the same method (including readings at 42 hours on 579 strains) for sensitivity to penicillin, tetracycline, chloramphenicol, and novobiocin. A number of strains, representing different patterns of resistance in the ditch plate test, were tested by tube dilution and plate dilution methods; doubling dilutions of the antibiotics were incorporated in nutrient agar or broth, and these were inoculated with undiluted and with a 1/500 dilution of overnight broth culture, one drop (0.02 ml.) in the tube test and a loopful in the plate test.

TABLE I

620 STRAINS OF *STAPHYLOCOCCUS AUREUS* ISOLATED DURING FOUR WEEKS OF 1957 SHOWING PATTERNS OF SENSITIVITY BY DITCH-PLATE TESTS READ AT 18 HOURS AND AT 42 HOURS

Sensitivity to Erythromycin*	Sensitivity to Oleandomycin								Sensitivity to Spiramycin							
	Test Read at 18 Hours				Test Read at 42 Hours				Test Read at 18 Hours				Test Read at 42 Hours			
	S	"S"	"R"	R	S	"S"	"R"	R	S	"S"	"R"	R	S	"S"	"R"	R
S	267	0	0	0	262	3	0	2	265	2	0	0	265	2	0	0
"S"	1	1	3	20	1	1	2	21	2	23	0	0	2	23	0	0
"R"	11	7	0	5	1	9	4	9	18	5	0	0	18	5	0	0
R	24	129	71	81	3	26	17	259	239	66	0	0	238	67	0	0
Total	303	137	74	106	267	39	23	291	524	96	0	0	523	97	0	0

* Readings of the test after 42 hours' incubation; these were the same as at 18 hours in all except seven strains, which appeared more resistant after the longer period of incubation. S = Zone of inhibition the same as that of sensitive control. "S" = Zone of inhibition narrower than that of sensitive control. "R" = Growth up to but not across antibiotic-containing ditch. R = Growth extends across ditch.

Growth curves of staphylococci from small inocula in broth with and without the antibiotics oleandomycin and erythromycin were constructed from series of viable counts made by the method of Miles, Misra, and Irwin (1938).

A selection of strains was phage-typed by the method of Williams and Rippon (1952).

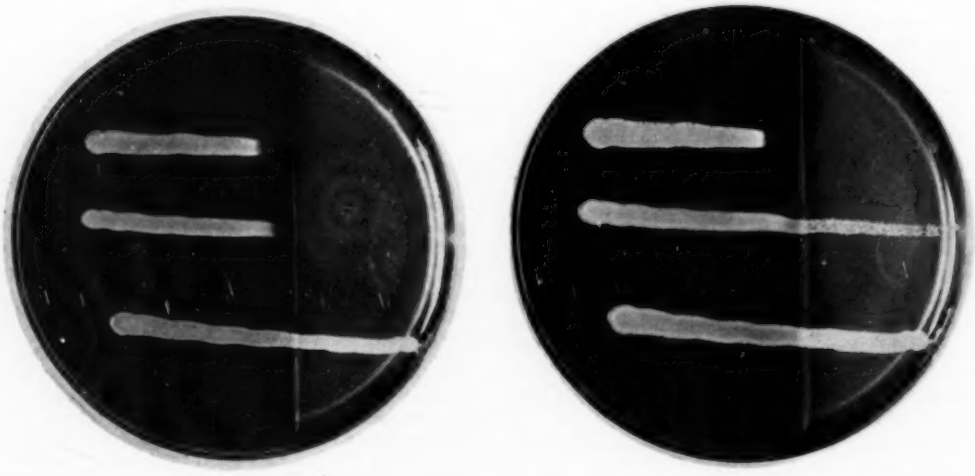
Habituation of a number of sensitive strains of *Staph. aureus* to growth in the presence of the antibiotics was carried out by serial transfer in fluid media, as described elsewhere (Lowbury, 1957).

Results

Table I shows the sensitivity to erythromycin, oleandomycin, and spiramycin by ditch plate tests of 620 strains of *Staph. aureus* isolated during a period of four weeks in 1957. The strains are classified as fully sensitive (S) or showing three degrees of resistance ("S," "R," and R) defined in the footnote to the Table.

Strains recorded as sensitive (S) to erythromycin were all fully sensitive to oleandomycin after 18 hours, and almost all remained fully sensitive after 42 hours' incubation. The same strains were almost always fully sensitive to spiramycin after 18 hours' and 42 hours' incubation.

Strains which showed more resistance to erythromycin fell into two groups: (*a*) the small number which were slightly resistant to erythromycin ("S") were nearly always fully resistant to oleandomycin after 18 hours' incubation, and usually slightly resistant ("S") to spiramycin. (*b*) Strains which were more resistant



18 hours 42 hours

FIG. 1.—An oleandomycin ditch plate test on three strains of *Staph. aureus* photographed after incubation for 18 hours (left) and 42 hours (right). The strain in the middle, which appears moderately sensitive at 18 hours but resistant at 42 hours, was resistant to erythromycin. Above it is a strain sensitive to erythromycin and oleandomycin, and below a strain resistant to both antibiotics.

to erythromycin ("R" and R) were usually intermediate in their resistance to oleandomycin ("S" or "R") after 18 hours' incubation, but had in most cases become fully resistant after 42 hours' incubation. About one quarter of these showed some degree of resistance to spiramycin. Spiramycin sensitivity was the same after 18 hours' and 42 hours' incubation of the test plates. The different patterns of sensitivity and resistance after 18 hours' and 42 hours' incubation of an oleandomycin ditch plate are illustrated in Fig. 1.

Tests carried out in parallel with this series of staphylococci on ditch plates containing tetracycline, penicillin, chloramphenicol, and novobiocin showed that heavy delayed growth of staphylococci across the ditch on continued incubation virtually never occurred with these antibiotics, or with spiramycin or erythromycin. On some plates there was some thin delayed growth across the tetracycline ditch, apparently due to the relative instability of tetracycline; this mechanism, however, cannot be invoked to explain the delayed growth of erythromycin-resistant staphylococci in the presence of oleandomycin, for no loss in potency of that antibiotic could be detected after 42 hours' incubation.

Staphylococci showing dissociated resistance to erythromycin were often found to grow over the erythromycin ditch—and, after 42 hours, over the oleandomycin ditch—in the form of semiconfluent growth or large discrete colonies even when the growth at a distance from the antibiotic ditch was confluent. This suggests that only a fraction of the organisms of such a strain are resistant to erythromycin and, on further incubation, to oleandomycin.

The separate tests of oleandomycin and spiramycin sensitivity which were made on staphylococci from burns during the greater part of 1957 were quite consistent with the results of the study summarized in Table I. One additional feature was the presence in a small number of burns of staphylococci more resistant to spiramycin than those recorded in the Table. Several of these were isolated from patients who had received spiramycin therapy and from other patients who were in the ward at the same time.

Table II shows the results of tube and plate dilution tests on 36 strains of *Staph. aureus*, 12 being fully sensitive (ES, OS, SS) and 12 resistant (ER, OR, SR) to erythromycin, oleandomycin, and spiramycin by ditch plate tests. The remaining 12 strains were, by the same criteria, resistant to erythromycin, sensitive to spiramycin, and moderately sensitive to oleandomycin (ER, O "S," SS). In all the tests two bacterial inocula, 1 in 1 and 1 in 500, were used. There was no appreciable difference between readings at 18 hours and at 42 hours, or between the results of tests with the different inocula, except in the case of oleandomycin. The minimal inhibitory concentration (M.I.C.) of this antibiotic was appreciably higher in tests done with larger inocula of strains ES, OS, SS, and ER, O "S," SS; only strains of pattern ER, O "S," SS showed a higher M.I.C. of oleandomycin when tests were read after 42 hours' than after 18 hours' incubation. There was no consistent difference in the results of tube and plate dilution tests.

Fig. 2 shows the effects of oleandomycin on the growth curves of three strains of *Staph. aureus*, one of each sensitivity pattern. In the presence

TABLE II

SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* BY TUBE AND PLATE DILUTION TESTS

Sensitivity Pattern by Ditch Plate Test*	Strains	Test	Bacterial Inoculum	M.I.C.† Erythromycin (μg. per ml.)		M.I.C. Oleandomycin (μg. per ml.)		M.I.C. Spiramycin (μg. per ml.)	
				18 Hours	42 Hours	18 Hours	42 Hours	18 Hours	42 Hours
ES, OS, SS	12	Tube	10⁰	0·4	0·4–0·8	1·6–6·25	1·6–6·25	6·25	6·25–50·0
			1/500	0·2	0·2–0·4	0·8	1·6	6·25–12·5	12·5–25·0
		Plate	10⁰	0·2	0·2–0·4	1·6–3·12	3·12–6·25	6·25–12·5	12·5–25·0
			1/500	0·2	0·2–0·4	0·4–0·8	0·8–1·6	6·25–12·5	12·5–25·0
ER, O "S," SS	12	Tube	10⁰	>100	>100	3·12–12·5	25–>100	6·25–25·0	12·5–25·0
			1/500	>100	>100	0·8–3·12	3·12–100	12·5	12·5–25·0
		Plate	10⁰	>100	>100	3·12–12·5	50–>100	6·25–12·5	12·5–50·0
			1/500	>100	>100	1·6–3·2	6·3–100	6·25	6·25
ER, OR, SR	12	Tube	10⁰	>100	>100	>100	>100	50–100	50–100
			1/500	>100	>100	>100	>100	25·0–50·0	50–100
		Plate	10⁰	>100	>100	>100	>100	50–100	100–>100
			1/500	>100	>100	>100	>100	100	100

* ES, OS, SS = "sensitive to erythromycin, oleandomycin, and spiramycin." For key to grades of resistance, see footnote to Table I.
† M.I.C. = minimal inhibitory concentration.

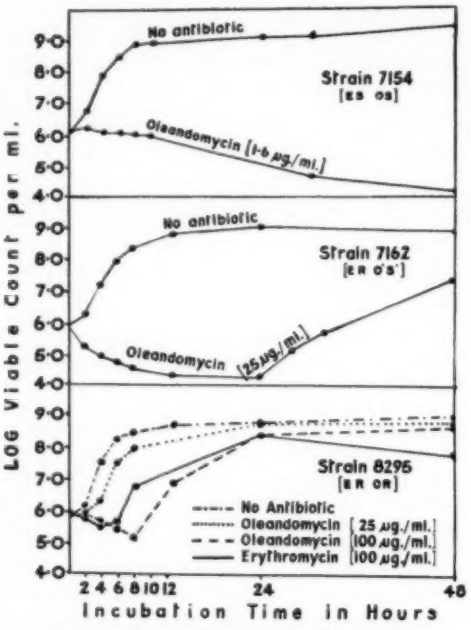


FIG. 2.—Growth curves of staphylococci showing sensitivity patterns (from above downward) ES, OS, ER, O "S," and ER, OR in the presence and absence of oleandomycin; in the lowest chart a growth curve of the staphylococci ER, OR in the presence of erythromycin is also presented. Note the long delay before growth of strain ER, O "S" began in the presence of oleandomycin.

of oleandomycin (1.6 µg. per ml.) the fully sensitive strain (ES OS) showed a progressive fall in viable count during the 48 hours of the test; the resistant strain (ER OR) exposed to oleandomycin, 25 µg. per ml., grew well, but in broth containing 100 µg. per ml. of oleandomycin (or erythromycin) there was an initial drop in viable count before growth started; the strain ER O "S" showed a drop in viable count during the first 24 hours, after which it grew well.

Phage Types of *Staph. aureus* Showing Different Sensitivity Patterns

Staphylococci showing the sensitivity pattern ES, OS, SS included a larger proportion of strains of phage group II than those showing dissociated or cross resistance to the erythromycin group of antibiotics; most of the latter showed various phage patterns belonging to group III, and in the series with sensitivity pattern ER, O "S," SS were several strains of type 80.

Cross-resistance after Habituation *in vitro*.—Attempts failed to obtain dissociated resistance to erythromycin (cf. Garrod, 1957) by daily subculture of sensitive strains in media containing increasing concentrations of erythromycin, and by exposing them for several days to a low concentration of the antibiotic (5 µg. per ml.) such as might be expected in wound exudate. By both methods resistance, when it emerged, was against oleandomycin as well as erythromycin. Most of 40 sensitive staphylococci tested which did not acquire resistance died on exposure for more than two days to 5 µg. erythromycin per ml.

In tests on the habituation of staphylococci to spiramycin considerable variation was found between strains in the degree of cross-resistance that appeared. For example, in Table III are shown the results after transfer of seven staphylococci of different phage types through 15 subcultures in the presence of spiramycin; three showed a high degree of cross-resistance to oleandomycin, two showed little cross-resistance, and one acquired little resistance to spiramycin itself or to the other antibiotics of the group.

TABLE III

ACQUIRED RESISTANCE OF *STAPH. AUREUS* ON HABITUATION TO SPIRAMYCIN (15 TRANSFERS)

Strain of *Staph. aureus* Phage Type	Increase* in Minimal Inhibitory Concentration of		
	Spiramycin	Erythromycin	Oleandomycin
42D	>32	>125	>32
51	64	250	>64
80	>32	>250	>32
42E	>32	4	16
52	32	64	4
47	4	8	2
47A	64	8	8

* Expressed as multiples of the initial minimal inhibitory concentration.

Cross-resistance with Other Antibiotics.—There was no consistent association in the resistance of staphylococci to erythromycin, penicillin, tetracycline, chloramphenicol, and novobiocin; the erythromycin-resistant staphylococci included a higher proportion of strains resistant to each of these antibiotics, presumably because they were all "hospital staphylococci" and reflected in their pattern of resistance the use of antibiotics in the ward.

RESISTANCE OF OTHER BACTERIAL SPECIES

Streptococcus pyogenes

During three years since we have used erythromycin, strains of *Streptococcus pyogenes* isolated from burns of four patients have been found resistant to erythromycin, in each case

during or after a course of erythromycin therapy for a streptococcal infection. The resistant strains have been of two serological types (3 and 14). Each of these strains has shown cross-resistance to oleandomycin and to spiramycin by a ditch plate test. On re-testing two strains by the plate dilution method, the M.I.C. of all three antibiotics was found to be >100 μg. per ml. The M.I.C. of sensitive strains is shown in Table IV.

TABLE IV

RELATIVE SENSITIVITY OF DIFFERENT WOUND ORGANISMS TO ANTIBIOTICS OF THE ERYTHROMYCIN GROUP

Antibiotic	M.I.C. (μg. per ml.) for 12 Sensitive Strains of		
	Staph. aureus	*Strep. pyogenes*	*Cl. welchii*
Erythromycin ..	0·2	0·05–0·025	0·2–0·1
Oleandomycin ..	0·8–0·4	0·4	3·2
Spiramycin ..	12·5–6·25	0·4–0·2	0·4–0·2

In the ditch plate test, resistant streptococci showed discrete colonies on the antibiotic ditch, although an undiluted broth culture was inoculated and gave confluent growth away from the ditch.

Clostridium welchii

One hundred strains of *Cl. welchii* from air, dust, and burns were tested by the ditch plate method, with 10 μg. per ml. of erythromycin and oleandomycin and 50 μg. per ml. of spiramycin in the ditch agar. All strains appeared sensitive to erythromycin and spiramycin and resistant to oleandomycin. All 24 strains of *Cl. tetani* tested at the same time were sensitive to all three agents.

Plate dilution tests were then made on 12 of the strains of *Cl. welchii* (see Table IV). These supported the findings of the ditch plate test, and showed *Cl. welchii* to be consistently more sensitive to erythromycin and spiramycin than to oleandomycin. This was in contrast to the pattern shown by sensitive staphylococci, which were least sensitive to spiramycin, and *Strep. pyogenes*, which showed approximately the same sensitivity to oleandomycin and to spiramycin.

PROPHYLAXIS AND THERAPY OF STAPHYLOCOCCAL INFECTION IN MICE

From the anomalous results of sensitivity tests reported above, it is impossible to predict whether infection by a staphylococcus showing the sensitivity pattern ER, O " S " is likely to respond to therapy by oleandomycin. The question can only be answered satisfactorily by controlled therapeutic trials in man, but we have tried to throw some light on this problem by a series of mouse protection tests.

Materials and Methods

Strains of *Staph. aureus*.—The three strains selected were the same as those used for the growth curves shown in Fig. 2, representing three resistance patterns, viz., 57/7154 (ES, OS, SS), 57/7162 (ER, O " S," SS), and 57/8295 (ER, OR, SR); they were originally isolated from burns.

Overnight broth cultures of these organisms mixed with an equal volume of mucilage of tragacanth (B.P.C.) were injected in 0.1 ml. amounts intraperitoneally into albino mice. After several passages to increase virulence, undiluted inocula were found to kill from eight to 10 out of 10 mice within 24 hours; none was killed by the 10^{-1} inoculum.

Prophylactic and Therapeutic Tests.—Groups of 10 mice were used.

In the prophylactic tests, staphylococci were injected intraperitoneally as described above. One group was left untreated, and the others received intramuscular injections of erythromycin (0.64 mg.), oleandomycin (0.8 mg.), and spiramycin (2.0 mg.); these weights of antibiotic were divided between four approximately four-hourly doses, the first being given at the time of the bacterial inoculation. The dosage was calculated to correspond, weight for weight, with the recommended human dosage. The number of animals surviving after 24 hours was recorded.

In the therapeutic tests the first dose of antibiotic was given two hours after the injection of staphylococci, and three further doses were given at four-hour intervals; the fully resistant strain was not included in the therapeutic experiment. Some of the tests were repeated with a higher dosage of oleandomycin (1.6 mg.). The number of mice surviving after 10 hours and after 24 hours was recorded.

Results

Table V summarizes the results of these experiments. In the prophylactic test, mice were fully protected by all three antibiotics against the sensitive staphylococcus (ES, OS, SS). Mice infected with the strain of pattern ER, O " S," SS were apparently well protected by spiramycin (10 out of 10 survivors), less well protected by oleandomycin (seven out of 10 survivors), and least well protected by erythromycin (four out of 10 survivors). Against infection with the resistant strain (ER, OR, SR), however, oleandomycin and spiramycin were less active than erythromycin.

In the therapeutic tests, six out of 20 mice treated with erythromycin and 13 out of 30 mice treated with oleandomycin (at two dosages) were alive 24 hours after infection with strain ER, O " S," SS compared with five out of 30 untreated controls which survived. The differences were not striking, but the result is consistent with findings in the prophylactic test, suggesting that oleandomycin has a small and erythromycin a

TABLE V

PROPHYLAXIS AND THERAPY OF STAPHYLOCOCCAL INFECTION IN MICE

Form of Treatment	Strain of *Staph. aureus*	Sensitivity Pattern	Hours After Infection	Proportion of Mice (Groups of 10) Surviving under Treatment with				
				Erythromycin (0·8 mg.)	Oleandomycin (0·64 mg.)	Oleandomycin (1·6 mg.)	Spiramycin (2·0 mg.)	No Antibiotic
Prophylactic ..	57/7154	ES, OS, SS	24	10	10	—	9*	1
	57/7162	ER, O " S," SS	24	4	7	—	10	2
	57/8295	ER, OR, SR	24	4	1	—	2	0
Therapeutic ..	57/7154 (i)	ES, OS, SS	10	10	10	—	10	2
			24	8	1	—	4	0
	,, (ii)	,,	10	10	—	10	—	8
			24	10	—	10	—	2
	57/7162 (i)	ER, O " S," SS	10	10	10	—	10	6
			24	4	2	—	10	0
	,, (ii)	,,	10	—	10	—	—	4
			24	—	4	—	—	1
	,, (iii)	,,	10	9	—	10	—	6
			24	2	—	7	—	4

* Only nine mice were included in this group.

smaller therapeutic action against strains with the sensitivity pattern ER, O " S "; spiramycin appeared to have a greater therapeutic value for infection by these organisms. All three agents were more active *in vivo* against fully sensitive staphylococci.

DISCUSSION

The delayed growth of erythromycin-resistant *Staph. aureus* in the presence of oleandomycin sets a new problem in the interpretation of sensitivity tests not encountered with the other antibiotics. If the results after 18 hours' incubation are accepted, many strains would be described as sensitive which a reading at 42 hours would classify as resistant. It seems possible that a staphylococcal infection might be arrested by this retardation of growth in the presence of oleandomycin, especially as the organisms which do eventually break through the oleandomycin barrier (like those which at an earlier stage grew in the presence of erythromycin) appear to represent a minority of those present in the bacterial inoculum. Animal tests suggest that oleandomycin (and even erythromycin) may have some prophylactic and therapeutic action against such staphylococci, but the action is smaller than that exerted by either agent against genuinely sensitive strains.

It would seem from the incomplete nature of the cross resistance of staphylococci to erythromycin, spiramycin, and oleandomycin that each of these antibiotics has more than one mechanism of action against sensitive bacteria; at least one mechanism is shared, but others are independent. The mechanism by which oleandomycin retards the growth of erythromycin-resistant staphylococci from burns, for example, is not shared with erythromycin; and spiramycin, which in these studies remained fully active against most of these erythromycin-resistant staphylococci, obviously has quite a different unshared mechanism of bacteriostasis. Spiramycin is the least active antibiotic of the group against sensitive *Staph. aureus*, and it seems also less closely related in this respect to the other two than they are to each other. Against *Cl. welchii*, on the other hand, oleandomycin appears to be considerably less active than the other antibiotics of the group. *Strep. pyogenes* is most sensitive to erythromycin (cf. Garrod and Waterworth, 1956), and equally sensitive to oleandomycin and spiramycin. These differences in the relative sensitivity of different bacterial species to the three antibiotics are further evidence of the complexity of the bacteriostatic mechanisms involved.

SUMMARY

Sensitivity to erythromycin, oleandomycin, and spiramycin was tested by a ditch plate method on 620 strains of *Staph. aureus* isolated from burns during one month, and a selection of these and other staphylococci were tested also by tube and plate dilution methods. The tests were recorded after 18 hours' and again after 42 hours' incubation. Growth curves of staphylococci in the presence and in the absence of oleandomycin were plotted from viable counts.

Of the 267 strains (43%) which were sensitive to erythromycin, all were sensitive to oleandomycin and 265 were sensitive to spiramycin in tests examined after 18 hours' incubation; slightly reduced sensitivity was shown to oleandomycin in three of these strains when the tests were examined after 42 hours' incubation. Of the 353 strains (57%) which were resistant or less sensitive to erythromycin, only 36 were fully sensitive to

oleandomycin in tests examined after 18 hours, 211 strains showing intermediate sensitivity and 106 being fully resistant, i.e., showing growth across the ditch; when the tests were re-examined after 42 hours' incubation only five strains were fully sensitive to oleandomycin; 59 showed intermediate sensitivity and 289 were fully resistant. This altered pattern of resistance on continued incubation was not shown in tests with spiramycin, to which 259 of the erythromycin-resistant strains were sensitive after 18 hours and 258 after 42 hours' incubation.

Growth curves showed that erythromycin-resistant oleandomycin-"sensitive" staphylococci began to multiply in the presence of oleandomycin after about 24 hours' lag, during which the number of viable organisms fell.

Mouse protection tests suggested that oleandomycin and even erythromycin might have some therapeutic action against staphylococci showing dissociated resistance to erythromycin, but the action was smaller than that of spiramycin against these strains or than any of the erythromycin group antibiotics against fully sensitive strains.

Spiramycin was the least active of these antibiotics against *Staph. aureus*, but oleandomycin was the least active against *Cl. welchii*. Against *Strep. pyogenes* oleandomycin and spiramycin seemed to have about the same bacteriostatic activity. For these reasons and because of the incomplete pattern of cross resistance of *Staph. aureus* it seems that each of these antibiotics has more than one mechanism of action.

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ISOLATION OF *CHROMOBACTERIUM PRODIGIOSUM* FROM EMPYEMA

BY

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Chromobacterium prodigiosum (*Serratia marcencens*) is an aerobic, motile, Gram-negative rod that produces a red pigment which is insoluble in water. This bacterium has generally been considered to be a saprophyte. It is usually found in water and soil and may contaminate food.

There are, however, in the literature reports of cases where *Chr. prodigiosum* has been isolated from pathological processes in men and animals. Patterson, Banister, and Knight (1952) have described a fatal septicaemia, Aronson and Alderman (1943) and Rabinowitz and Schiffrin (1952) meningitis, Hawe and Hughes (1954) bacterial endocarditis, and Wheat, Zuckerman, and Rantz (1951) reported 11 cases of urinary tract infections due to *Chr. prodigiosum*. In 1913 Woodward and Clarke reported a case of a patient who had a chronic cough, probably from bronchiectasis. He brought up "bloody" sputum, but red blood cells were not found in the sputum. Cultures of the sputum grew *Chr. prodigiosum*. It seems obvious that the red colour of the sputum was due to the red pigment of the organism. Robinson and Woolley (1957) and Gale and Lord (1957) reported similar cases. Also Déom and Mortelmans (1953) described a fatal infection with *Chr. prodigiosum* in a colt.

Case Report

A man, aged 55, was admitted to hospital in February, 1957, with right spontaneous pneumothorax. In 1948 this patient had had a left spontaneous pneumothorax. With continuous suction the lung re-expanded in 24 hours, confirmed radiologically. Two days later fluid was present in the right costophrenic angle and the lung collapsed. The fluid was aspirated but not cultured. Thoracoscopy was performed on April 3, 1957. On April 25 the patient was admitted to the surgical clinic with extreme dyspnoea. Intercostal drainage was established with continuous suction. On May 7 "blood-stained" fluid drained through the tube, and the patient started to have pyrexia up to 38.5° C. The fluid was not cultured. To secure a better dependent drainage of the chest, a self-retaining catheter was introduced into the sixth intercostal space anterially. The "blood-stained" fluid draining from this catheter was cultured on May 28 and *Chr. prodigiosum* in pure culture was isolated. Two further cultures on June 8 and July 1 yielded *Chr. prodigiosum* and a few colonies of *Pseudomonas aeruginosa*.

On June 10 chlortetracycline, 1.5 g. per day, was begun orally, but the temperature remained high. Following a sensitivity test chloramphenicol was instilled into the pleural cavity. The temperature gradually fell and the lung started to re-expand. By August the lung had re-expanded, but a small empyema cavity remained at the right base. Cultures on August 8 and September 14 yielded *Pseudomonas aeruginosa*.

Bacteriology

The organism isolated was a Gram-negative, motile, short rod with occasional longer rods. It formed round, convex colonies with a smooth, shiny surface on nutrient agar and blood-agar plates. It grew at 37° C. as well as at 25° C. It produced orange-red pigment on agar at 25° C., which deepened after three to four days, but at 37° C. gave little or no pigment. The red pigment was insoluble in water, but soluble in alcohol and in chloroform. An alcoholic solution of the pigment turned bright pink on the addition of hydrochloric acid and yellow on the addition of alkali. On blood-agar pigment was produced at 25° C. and there was haemolysis. The organism grew on MacConkey medium, but the colonies were smaller and produced deep red pigment on standing at room temperature. It produced dense turbidity in broth at 25° C., with a pink ring. It produced clot in litmus milk, followed by liquefaction. Saccate liquefaction was caused in a gelatin stab and a deep red

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pigment produced. The organism liquefied Löwenstein-Jensen and Loeffler's media. Other reactions were as follows: Indole −, M.R. −, VP +, Koser's citrate +, nitrates reduced to nitrites; NH_3 production +, catalase +, methylene blue reduced, urea − at 24 hr., H_2S (on Kligler's medium) −, phenyl-pyruvic acid − (Henriksen), K.C.N. (Möller test) +, malonate −, amino-acid decarboxylases; lysine +, ornithine +, glutamic acid −, arginine −. It produced acid and a little gas from glucose, maltose, sucrose, mannitol, and salicin at 37° C., but not from lactose, dulcitol, or xylose.

The description of this organism resembles that of *Chromobacterium prodigiosum* given by Wilson and Miles (1955).

Sensitivity to Antibiotics.—The isolated strain was sensitive by the paper disc method to streptomycin, chloramphenicol, and neomycin, and resistant to penicillin, oxytetracycline, chlortetracycline, tetracycline, and erythromycin.

Pathogenicity for White Mice. — The pathogenicity of the isolated organism for mice was determined as follows:

Groups of five mice were injected intraperitoneally with 0.2 ml., 0.1 ml., or 0.05 ml. of a 24-hour broth culture. All five died after 0.2 ml., three after 0.1 ml., and one after 0.05 ml. All mice survived injection of 0.2 ml. of a 1 in 10 dilution of a 24-hour broth culture. Five mice were injected with 0.05 ml. of the 24-hour broth culture. At 24 hours only one mouse was dead. The other mice survived. Four groups of four mice each were inoculated with 0.2 ml. of serial dilutions (10^{-1} to 10^{-4}) in peptone water of the above 24-hour broth culture. All the mice survived. The number of viable organisms inoculated was counted by the method of Miles and Misra (1938) and the lethal dose of this organism for mice was estimated to be >11,000,000 bacteria.

Discussion

In the textbooks of bacteriology *Chr. prodigiosum* has been described as a saprophyte. But there seems to be little doubt, from the published reports, that this organism can produce mild or fulminating fatal illness. Gale and Lord (1957) observed in mice enhancement of the virulence of a strain of *Chr. prodigiosum* when injected with mucin. It has been noted by many workers that the virulence of an organism will be enhanced by mucin only if the organism is pathogenic or at least weakly pathogenic.

In the present case the same organism, *Chr. prodigiosum*, was isolated three times over a period of 30 days. In the first culture (May 29) of the empyema fluid, *Chr. prodigiosum* was isolated in pure culture. In the other cultures (June 8 and July 1) a few colonies of *Pseudomonas aeruginosa* were isolated in addition to the profuse culture of *Chr. prodigiosum*. It is difficult, therefore, to assess with certainty the pathogenicity of *Chr. prodigiosum*. However, the isolated strain was weakly pathogenic for mice. Moreover, the development of the infection suggests strongly the pathogenicity of the organism, while the subsidence of the fever was accompanied by disappearance of *Chr. prodigiosum* but not of the *Ps. aeruginosa*.

The beneficial therapeutic result following the instillation of chloramphenicol into the pleural cavity should be noted. This observation is in accordance with the antibiotic sensitivity test *in vitro*. Robinson and Woolley (1957) and Béguet (1953) reported the sensitivity *in vitro* to chloramphenicol of *Chr. prodigiosum*. Also Hawe and Hughes (1954) thought that either chloramphenicol or neomycin might offer the best chance of successful treatment.

Summary

The isolation of a strain of *Chr. prodigiosum* from empyema fluid is presented. A description of this strain is given.

We wish to thank Dr. M. T. Parker, of the Public Health Laboratory, Manchester, who confirmed the identity of the strain of *Chr. prodigiosum* and kindly performed the amino-acid decarboxylases, K.C.N., P.P.A., and malonate tests.

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ADENOMATOID HAMARTOMA OF THE LUNG IN A NEWBORN INFANT

BY

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In pulmonary pathology the term "hamartoma" is most commonly applied to a group of tumours in adults which usually contain cartilage as a constituent and arise from connective tissue (Sutherland, Aylwin, and Brewin, 1953). Willis (1958) does not think such growths are named correctly and gives good reasons for regarding them as acquired mixed tumours. On the other hand the mass replacing the entire upper lobe of the right lung of a newborn infant reported by Jones (1949) does seem to have been a true hamartoma in which interstitial elements predominated.

Another type of hamartoma was described by Ch'in and Tang (1949) as "congenital adenomatoid malformation." These authors considered their case to be the first in the English language, there being 10 previous reports in German. The condition, however, could be included in the category of "mixed tumours" as defined by Womack and Graham (1938) and undoubted examples were reported by Harris and Schattenberg (1942) under the names "anlagen and rest tumours." Thomas's case (1949) of "cystic hamartoma" in a newborn infant also falls into this group, and so does the "diffuse hamartoma" of the left upper lobe of a girl aged 7½ weeks which was successfully removed at thoracotomy and reported by Graham and Singleton (1955).

An example is presented here in a newborn premature infant of this second type of hamartoma which involved the whole of the right lung whilst the left lung also showed imperfect development.

Case Report

Clinical History.—The mother had lost her first infant when it was 9 days old from meningitis. She was aged 24 at the time of the present pregnancy, and the expected date of delivery was April 27, 1958. When seen on January 19, 1958, she had a slight urinary tract infection with pain under the right subcostal margin and a trace of albumin in the urine. There was no toxaemia and she felt plenty of foetal movements, but hydramnios was present and the uterus was the size of a 38 weeks' pregnancy. Labour began spontaneously on January 21, and a few hours later she delivered herself of a female child. The placenta was said to be ragged and was adherent to the anterior uterine wall, needing manual removal. After this the mother's progress was satisfactory.

The baby was cyanosed, weighed 2 lb. 14 oz., and only lived for 30 minutes. Its appearance suggested hydrops. Both mother and baby were blood group A Rh positive, and the direct Coombs test on the cord blood was negative.

Necropsy Findings.—No abnormalities were found in any of the infant's systems or organs except the lungs.

FIG. 1.—General view of thoracic viscera. (The two horizontal cuts in the right lung were made at necropsy.)

Right Lung.—This was represented by a tumour filling most of the thoracic cavity (Fig. 1). The surface of the mass had a mosaic pattern from numerous very shallow sulci. An oblique fissure, 0.5 cm. deep, ran across most of the anterior aspect, out there was no true division into lobes. On section the mass was found to consist of firm, solid, pale tissue with numerous irregularly shaped empty cavities (Fig. 2). The biggest cavity was 1.1 cm. in diameter, but the majority were small, averaging about 0.2 cm. in diameter. The main bronchus had only two subdivisions which ended blindly at the tumour. The blood supply came from the pulmonary artery, and whilst some vessels entered the hilum of the tumour others ramified over the surface like a pia mater and sent penetrating branches into the underlying substance. Venous drainage was into the left atrium.

Left Lung.—This was small and vestigial. The left bronchus divided into two branches, but the lung itself was not subdivided. The arrangement of the major blood vessels was normal.

Histology.—The main bronchi on each side appear normal. Slides prepared from 10 different parts of

FIG. 2.—Part of cut surface of tumour and heart.

FIG. 3.—General view of tumour tissue. Haematoxylin and eosin ×73.

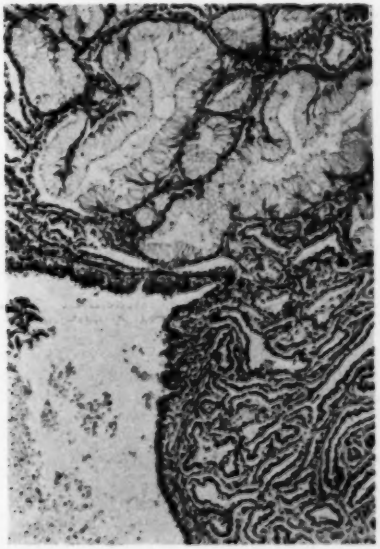

FIG. 4.—Edge of a cyst showing mucous glands in top half of field. Haematoxylin and eosin, ×73.

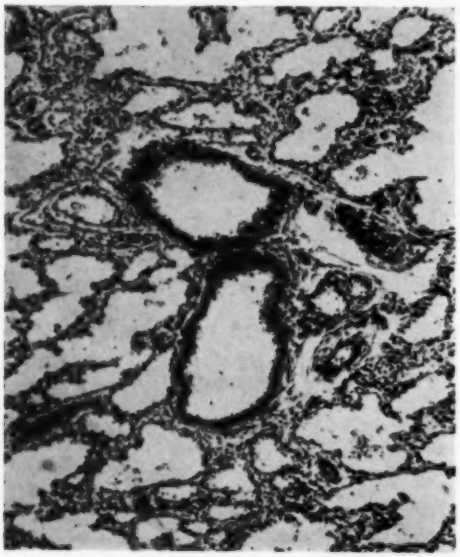

FIG. 5.—Subpleural part of tumour showing resemblance to congenital alveolar dysplasia with structures resembling bronchioles. Haematoxylin and eosin, ×73.

the right lung mass show it to consist of a loose scanty mesenchymal stroma riddled with compact glandular spaces of various sizes and shapes, most of them small but some large and cystic (Fig. 3). The lining cells are predominantly columnar and occasionally ciliated, with clear cytoplasm giving negative stains with periodic-acid-Schiff and mucicarmine. The nuclei are centripetal. Occasionally the lining of a cyst is slightly papillary. In the mesenchyme around and between the spaces are occasional strands of plain muscle, some elastic fibres, and a few collagen bundles. Some of the largest cysts are associated with small collections of mucous glands (Fig. 4) giving a positive reaction with periodic-acid-Schiff and mucicarmine. Blood vessels are few. Towards the pleura the spaces tend to become more regular and approach the appearances of the alveoli in congenital alveolar dysplasia (MacMahon, 1948), and structures resembling bronchioles are found here (Fig. 5) with very rarely a tiny piece of cartilage.

The small left lung has an excess amount of interstitial tissue and gives the picture of congenital alveolar dysplasia although it is rather poorly vascular.

The liver shows marked haemopoiesis.

Discussion

Ch'in and Tang stressed the frequency of hydramnios in the mother and anasarca of the infant in these cases and ascribed it to mechanical interference with the foetal circulation. We would agree with this explanation.

Although not common the condition is a fairly clearly defined entity and many synonyms have been applied. It is related to congenital cystic disease of the lung on one hand and congenital alveolar dysplasia on the other. With its obvious overgrowth and clear origin from a developmental anomaly it falls within the definition of a hamartoma, and to emphasize its glandular structure we propose for it the name "adenomatoid hamartoma."

Bowden (1948) brought forward strong evidence that congenital cystic disease of the lung is a malformation, and that arrest of development of the bronchial tree is the important aetiological factor. Thomas assigned to his tumour a developmental age corresponding to approximately the nineteenth week of foetal life. MacMahon suggested that congenital alveolar dysplasia has its origin dating back to the tenth and twelfth week of intra-uterine life since it bears a resemblance to the histological pattern of a 3- to 4-month old foetus at which stage the immature lung is rich in mesenchyme. According to Patten (1953) the right primary bronchus gives rise to two lateral bronchial buds during the fifth week. In our case the main right primary bronchus had only given rise to one bud, and it would therefore appear that the error dated back to the fifth week of gestation.

Summary

A case is presented of a newborn premature infant with a true hamartomatous tumour of the lung in which epithelial structures predominated. The error appears to have dated back to the fifth week of gestation.

Our thanks are due to Professor R. A. Willis for much helpful advice, and to Mr. G. Tulloch and Mr. J. R. Lusby for assistance with technique and photography.

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"MALIGNANT LEUKOPLAKIA" OF THE RECTUM: METAPLASTIC BASO-SQUAMOUS CARCINOMA OF THE RECTAL MUCOSA

BY

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In 1949 Dukes described an unusual condition which he called "leukoplakia of the rectum, resembling carcinoma." The clinical features and the naked-eye appearance of the lesion were those of carcinoma, but this diagnosis was disproved on histological examination, which showed that the rectal mucosa had been completely replaced by hyperplastic squamous epithelium. The patient, a middle-aged man, was a lunatic, and, for want of other evidence, the epithelial changes were attributed to the practice, common amongst lunatics, of harbouring foreign bodies in the rectum. This case appears to be the only one of its kind in the literature.

We report a second example of squamous-cell metaplasia of the rectum which differs from the other in that the squamous epithelium not only looked malignant but had in fact undergone malignant change, the growth being a carcinoma of mixed squamous and basal cell type.

Clinical History

A woman, aged 47, was admitted to hospital complaining of rectal discomfort for two weeks and loss of appetite and weight for an indefinite period. Bowel action had always been regular. Examination revealed a stricture 4 in. above the anus and a large ischio-rectal abscess.

The abscess was opened and found to communicate with the rectum at the site of the stricture. A faecal fistula, which developed subsequently, was treated by left iliac colostomy. Biopsy material from the site of stricture showed squamous carcinoma, and the rectum was therefore removed two months later, when the patient's general condition had improved. No intra-abdominal metastases were felt at operation and the primary growth seemed to have been completely eradicated. Eight months later, however, when the perineum was opened to drain a pelvic abscess, it was found that the tumour had recurred locally. Radiotherapy failed to arrest the growth and the patient died of malignant cachexia 16 months from the time of her first admission to hospital. Permission for a necropsy was refused.

Laboratory investigation during the course of her illness included Wassermann and Kahn tests and repeated Frei tests for lymphogranuloma venereum. All were negative.

Operation Specimen of Rectum and Anus (Fig. 1).—The mucosa in the upper two-thirds was thin and glazed, with a few small shallow ulcers and

FIG. 1.—The rectum removed at operation, showing the tumour at the lower end.

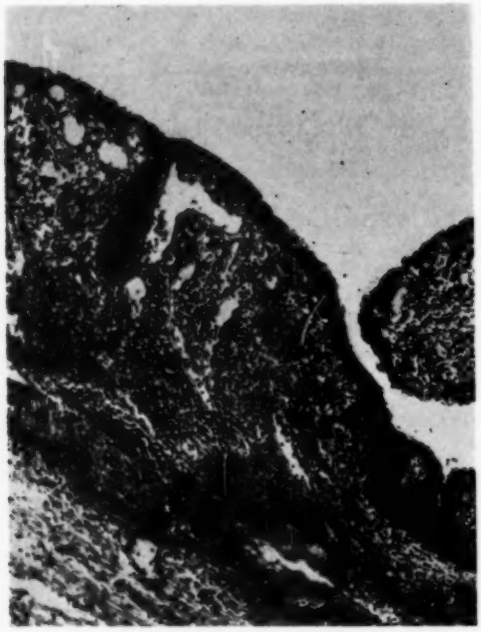

FIG. 2.—Thin, atrophic, metaplastic squamous epithelium in the upper part of the rectum (×75).

FIG. 3.—Keratinized hyperplastic squamous epithelium above the tumour (×75).

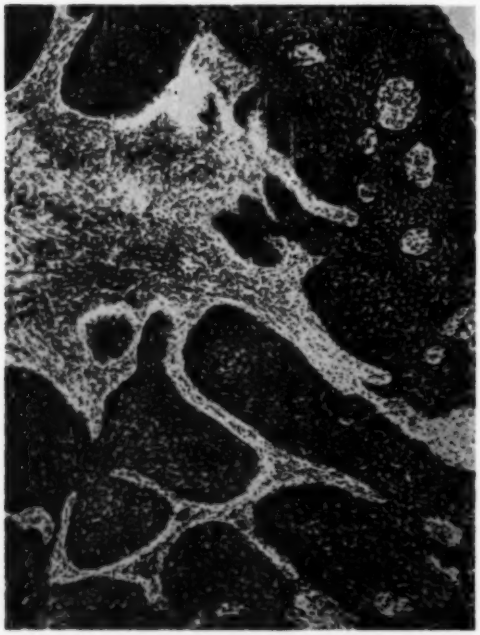

FIG. 4.—An area of tumour showing the pattern of basal cell carcinoma (×75).

FIG. 5.—An area of tumour showing the pattern of squamous cell carcinoma with epithelial pearl formations (×110).

FIG. 6.—Below the tumour: junction of normal columnar cell mucosa (bottom) with metaplastic squamous epithelium (top) (×150).

occasional white opaque thickened areas. Lower down, the mucosa became increasingly thickened in a pattern of pearly-white nodules and ridges merging into a fungating tumour which encircled the rectal wall, about 3 in. above the anus. Section at this level showed firm, white tumour spreading from the mucosa into the muscle layer.

Microscopical Examination.—The glandular mucosa was entirely replaced by squamous epithelium, thin and atrophied in the upper part of the specimen (Fig. 2), thick, hyperplastic, and heavily keratinized lower down (Fig. 3). In the neighbourhood of the tumour, atypical cells and mitotic figures became plentiful and irregular hyperplastic areas appeared in the basal and superficial layers of the epithelium. The tumour itself (Figs. 4 and 5) was a mixture of squamous and basal cell carcinoma, both elements being present as *in situ* and invasive forms. Tumour cells had infiltrated into the muscle of the rectal wall but were not seen in lymphatics or peri-rectal lymph nodes. Below the tumour the lining epithelium resumed the metaplastic squamous form, which in turn gave place to normal columnar cell mucosa (Fig. 6). The inflammatory element in the lesion was low-grade and chronic: superficial granulating ulcers on the mucosa and fibrosis in the submucosa.

Discussion

The histological findings in this case leave no doubt that the carcinoma arose in pre-existing squamous epithelium and that the primary lesion is similar to that described by Dukes (1949).

In defining the carcinoma as a secondary phenomenon, malignant disease can be excluded as a cause of the leukoplakia. This is necessarily the first step in attempting to elucidate the genesis of the lesion, since malignant disease is the only condition in which squamous change in gastro-intestinal epithelium is a recognized, albeit rare, occurrence (Hicks, 1953; Schmidtmann, 1919; Hicks and Cowling, 1955; LeBlanc, Buie, and Dockerty, 1950). As for the other known metaplasia-inducing agents (setting aside their seeming ineffectiveness in the gastro-intestinal tract), there is none which can be identified satisfactorily with the rectal lesion.

Mechanical Irritation.—Trauma caused by a foreign body cannot perhaps be altogether discounted on a patient's denial of perverted habits. But even if this form of mechanical irritation could be established, there is insufficient evidence available for assessing its effect on rectal mucosa. Rectal prolapse, another possible source of mucosal traumatization, is subject to the same difficulty of proof. In experimental animals, rectal prolapse may cause squamous metaplasia and carcinoma (Wells, Slye, and Holmes, 1938), but it is not known to have this effect in man.

Chronic Inflammation.—This can almost be excluded on the short clinical history, and it is worth recalling that squamous metaplasia has not been observed in peptic ulcer, dysentery, colitis, or proctitis, no matter how chronic.

Congenital Malformation.—This is unlikely to leave squamous rests in the rectal mucosa since the rectum completes its development from hind gut before reaching the anal skin. Moreover, there are no reports of congenital squamous rests having been found anywhere in the gastro-intestinal epithelium. Finally, there is an ill-understood form of squamous metaplasia which occurs apparently fortuitously in adult life at such diverse and unexpected sites as bursae (King, 1950), pituitary (Biggart, 1949; Hunter, 1955), uterine tube (Plaut and Dreyfuss, 1940), or peritoneum (Crome, 1950). These lesions have all been small inactive foci not subject to malignant change. For the present, it would seem that they resemble rectal leukoplakia only in the mystery of their origin.

Experience with this case prompts the following observations.

(1) The cause of squamous-cell metaplasia of the rectum is unknown, but it is best regarded as a pre-cancerous state. Malignant change, though perhaps not inevitable, can certainly supervene.

(2) Even when benign, the lesion may be indistinguishable from carcinoma clinically (Dukes, 1949).

(3) Apparently reassuring biopsy reports of simple hyperplastic squamous epithelium should not deflect the surgeon from his proper course of action, which is excision of the rectum.

Summary

A case of baso-squamous cell carcinoma of the rectum associated with, and believed to be secondary to, widespread squamous metaplasia of the rectal mucosa (leukoplakia of the rectum) is described.

We would like to thank Dr. C. E. Dukes for his courtesy in discussing his case with us.

The photography is the work of Dr. T. C. Dodds, F.I.M.L.T., Head of the Medical Photography Unit, Edinburgh University.

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J. clin. Path. (1959), **12**, 179.

ON THE HISTOPATHOLOGY OF CHONDRODERMATITIS NODULARIS HELICIS CHRONICA

BY

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(RECEIVED FOR PUBLICATION JULY 4, 1958)

The histological features of chondrodermatitis have been well described in recent papers by Carol and van Haren (1941), Newcomer, Steffen, Sternberg, and Lichstenstein (1953), Shuman and Helwig (1954), and McConnell (1957). The epidermis shows acanthosis, with irregular broadening and deepening of the dermal papillae and hyperkeratosis with keratotic plugging of the follicles. The epithelium is often ulcerated, with fibrinous material in the base ; deep to this round-celled infiltration extends down into the corium where there is a proliferation of fibrovascular tissue. The latter reaction may be so marked as to resemble a vascular hamartoma. The perichondrium may be thickened or be involved by the down growth of the vascular granulation tissue. The underlying cartilage, often normal, may show degenerative changes or even necrosis due to direct involvement in the ulcer.

Although the histopathology of this condition has been repeatedly studied, several features and symptoms draw attention to its similarity to the glomus tumour. Pain in the helix appeared to be promoted not only by pressure but also by alteration in the temperature of the part. Also a painful cutaneous nodule, in an area where there are arteriovenous anastomoses, is almost diagnostic of a glomus tumour. These points suggested a re-examination of the histological specimens of patients already treated, and a study of new cases by more suitable histological techniques.

In the past four years, 21 cases referred by dermatologists have been seen. Of these, 18 have

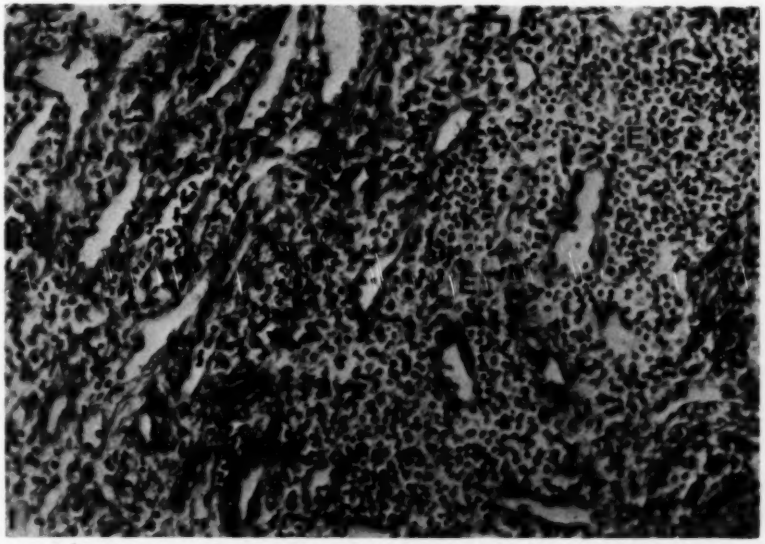
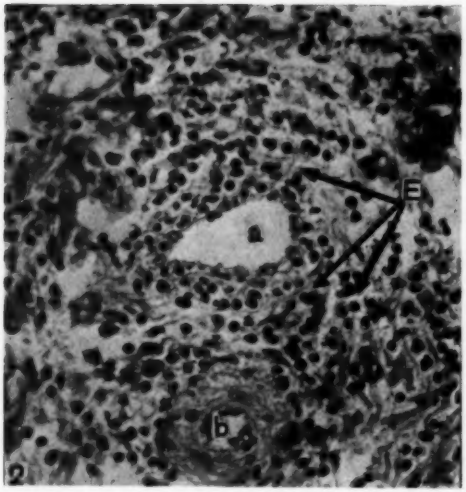
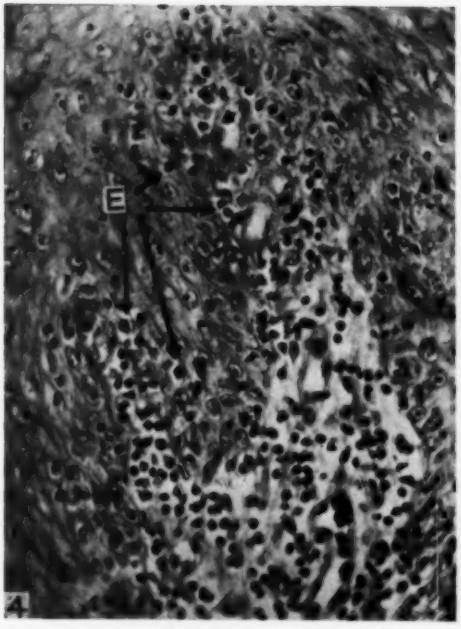


FIG. 1.—Papillary layer of the dermis in a case of chondrodermatitis helicis. Extensive infiltration of epithelioid cells (E). Mallory-Azan; × 220.





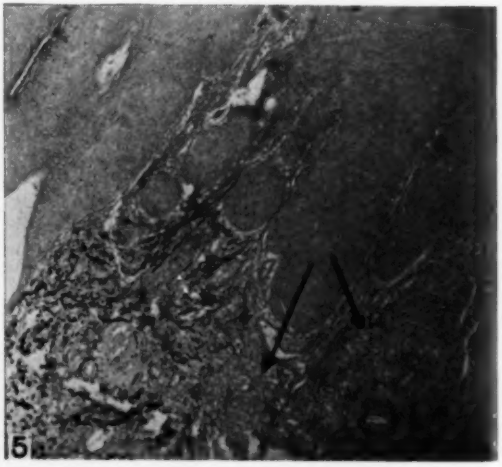


FIG. 2.—Artery (a) with epithelioid differentiation of the media (probably intermediate portion of an arteriovenous anastomose). (b) Normal arteriole for comparison. Note the proliferation of epithelioid cells (E) in the surrounding tissue and the distinctive clear cytoplasm of these. Mallory-Azan; × 290.

FIG. 3.—A clump of epithelioid cells: note the fine stroma. Mallory-Azan; × 800.

FIG. 4.—Infiltration of epithelioid cells (E) in the deeper layers of the stratum Malpighi. Mallory-Azan; × 290.

FIG. 5.—The normal network of elastic fibres is absent in the areas of epithelioid cell proliferation (arrows). Weigert; × 50.

been treated, in one the lesion resolved spontaneously, one refused further treatment having had three lesions excised previously, and one has been retained for observation on the natural history of the complaint.

Material and Methods

The present study includes 21 cases, diagnosed as chondrodermatitis, of which four have been examined by serial section. Specimens were fixed in formol-saline, Susa, or alcohol. Paraffin sections 6 μ thick were stained with Mallory-Azan, Masson, haematoxylin and eosin, Unna-Taenzer and Weigert for elastic tissue. The method of Cajal-De Castro for nerve fibres was also used.

Personal Observations

The accepted histological features were confirmed, but in three-quarters of the cases clear evidence was found of an unusual proliferation of epithelioid cells of the walls of arteriovenous anastomoses, a finding characteristic of glomus tumours of the type described by Masson (1924) in the finger tips.

The affected area is very rich in vessels with small lumina (Fig. 1), mainly arterioles, the walls of which are formed by stratified layers of epithelioid cells. It is often possible to ascertain that such proliferation initiated from the wall of these vessels and then flared out into the surrounding tissue (Fig. 2). There are also areas in which epithelioid cells have assumed the usual infiltrative appearance of a tumour ; they may be arranged in clumps or in palisade formation with a fine network of stromal tissue (Fig. 3). This proliferation mainly occupies the superficial layer of the dermis or the dermal papillae with little tendency to infiltrate the cartilage. Migration of epithelioid cells from the papillae into the superficial layers of the epidermis has also been observed (Fig. 4). In several cases accompanying inflammatory and degenerative changes were noted: round cells and localized areas of mucoid degeneration may be found scattered among the glomus tumour cells, mainly in the superficial layers. Such inflammatory infiltration may completely mask the presence of epithelioid cells and so lead to an incorrect conclusion.

Epithelioid cells are readily recognized in sections stained by the Mallory-Azan method. The normal network of elastic fibres in the dermis is absent in the zones occupied by solid clumps of epithelioid cells (Fig. 5). Nerve fibre stains did not show any unusual pattern in the innervation of the skin. The rate of proliferation of these cells does not appear to be very high, for mitoses are extremely rare. Ulceration of skin seems to be related to the lack of blood supply due to tumour infiltration in the underlying papillae, and hence degenerative changes in the epidermis and in the cartilage could be regarded as secondary.

Discussion

There is no doubt that the cellular proliferation observed in many cases of chondrodermatitis, reported here, has to be regarded as a typical proliferation of the specific cells present in the wall of arteriovenous anastomoses, which have been demonstrated in the human ear by Prichard and Daniel (1956). Their peculiar characteristics are readily recognized when suitable staining methods are employed. It follows that when epithelioid cells proliferate in this fashion, the lesion should be classified under the heading of "glomus tumour." It should not be inferred that all cases of chondrodermatitis are to be considered glomus tumours of the helix, but some cases can be associated with the presence of a small glomus tumour and the symptoms are likely to be due to the latter.

Cases of glomus tumour of the auricle have been recorded by Opitz (1954), Ratzenhofer (1941), Sannicandro (1936), and Ertl (1943). None of these had been previously classified as chondrodermatitis, most likely because of their size (which was larger than the usual nodule) or because of their different situation (neither on the helix margin nor the antihelix).

The only report in which the association of chondrodermatitis and glomus tumour is clearly demonstrated is that of Fernández and Monserrat (1931), who described one case in a man aged 38. These authors concluded that this case was one of glomus tumour and felt that all cases of chondrodermatitis should be so classified. Stout (1935) did not accept the diagnosis, while Freudenthal, Anderson, and Weber (1937) commented, "This case is apparently a chondrodermatitis nodularis helicis and not a glomus tumour," without any further explanation. The opinion of Fernández and Monserrat, however, is supported by photomicrographs which show a proliferation of epithelioid cells. Their interpretation was correct for that case, but we are unable to share their enthusiasm in advocating the classification of chondrodermatitis as glomus tumour on every occasion. Only certain of the cases examined, although a majority, exhibited evidence of proliferation of epithelioid cells for which the diagnosis of glomus tumour is justified.

Summary

Twenty-one cases of chondrodermatitis nodularis helicis chronica are reviewed. In more than three-quarters of these cases there is evidence of extensive proliferation of epithelioid cells from the wall of arteriovenous anastomoses, and therefore such cases have to be considered as small glomus tumours of the helix.

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ARGENTAFFIN CARCINOMA (CARCINOID TUMOUR) OF OVARY

BY

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(RECEIVED FOR PUBLICATION JULY 22, 1958)

Carcinoid tumours arising in cystic teratomata of the ovary have been observed by Stewart, Willis, and de Saram (1939), Gabrilove (1941), Blackwell and Dockerty (1946), Mitchell and Diamond (1949), Martin, Féroldi, and Cabanne (1956), and Falkmer (1956). A carcinoid tumour developing in a cystic teratoma of the testis was noted by Simon, McDonald, and Culp (1954).

Hartz (1945), who described a massive virilizing arrhenoblastoma containing cysts and collections of argentaffin-positive cells, interpreted the cyst lining as intestinal epithelium and the large and small groups of silver-reducing cells as carcinoid tissue. It is doubtful, however, whether this growth is acceptable as an ovarian argentaffin carcinoma. Dilated tubules and cysts lined by the mucinous epithelium are not uncommon findings in arrhenoblastoma of intermediate morphology, and, despite the presence of goblet cells and, sometimes, cells exhibiting a silver-reducing capacity, such structures necessarily are not endodermal in nature; they represent, more likely, metaplastic reactions in epithelial elements derived from the mesenchymal substance of the ovary. The interstitial cells in the tumour reported by Hartz exhibited a silver-reducing pigment, and it is probable that the tissue regarded as argentaffin was composed of large and small clusters of such interstitial cells.

If Hartz's case is excepted then the instance described below represents the eighth report of an argentaffinoma arising primarily in the ovary (Table I).

TABLE I

REPORTED CASES OF PRIMARY ARGENTAFFIN CARCINOMA OF OVARY

Author	Age of Patient in Years	Brief Clinical Notes	Tissue of Origin
Stewart *et al.* (1939)	68	Diarrhoea with blood and mucus for 5 months	Intestinal derivatives
	48	Abdominal pain 3 years. Mass in abdomen	Intestinal derivatives
Gabrilove (1941)	29	Incidental finding at necropsy	Gastric mucosa
Blackwell and Dockerty (1946)	59	Abdominal pain 1 year. Mass in pelvis	Uncertain, probably gastro-intestinal
Mitchell and Diamond (1949)	63	Generalized skin eruption. Radiograph showed calcified mass in pelvis	Respiratory epithelium
Martin *et al.* (1956)	72	Pain in abdomen and lumbar region. Mass in abdomen	Intestinal derivatives
Falkmer (1956)	39	Symptomless	Respiratory tract epithelium

Summary of Clinical Features

On December 5, 1956, the patient, aged 62 years, and menopausal since the age of 52 years, came to hospital complaining of lassitude, urticaria, pains in both shoulders, flatulence, abdominal discomfort, and loss of weight unaccompanied by an impaired appetite. She also admitted to a return of the "hot flushes" sometimes initiated by drinking a small quantity of whisky) which she had experienced at the climacteric. Her symptoms had been present for 12 months.

The cardiovascular and respiratory systems showed no abnormality. A pelvic tumour was found.

At operation on December 7 a large, partly solid cyst was discovered arising from the left ovary and this was removed without difficulty or subsequent complication. On palpating the abdominal organs the surgeon (Sir Arthur Gemmell) noted a nodule, the size of a shirt button, on the surface of the right lobe of the liver.

The patient was seen again on March 25 and 29, September 23, 1957, and January 9, 1958. She felt much better and admitted to having gained one stone in weight and that her "hot flushes" were much less marked.

Physical and radiographic examinations on these occasions revealed no respiratory, cardiovascular, abdominal, or skeletal abnormality. The electrocardiogram was normal and a follow-through barium meal showed no evidence of a gastro-intestinal lesion.

Since the diagnosis of the ovarian growth as a carcinoid tumour, the urine was repeatedly examined for 5-hydroxyindoleacetic acid; such tests, carried out by a qualitative chromatographic technique, have been consistently negative.

FIG. 1

FIG. 1.—Pseudomucinous epithelium lining inner wall of cyst. Haematoxylin and eosin × 95.

FIG. 2.—Occasional argentaffin cell in pseudomucinous epithelium. × 250.

FIG. 3.—Section through poriferous portion of cyst wall. Haematoxylin and eosin × 85.

FIG. 2

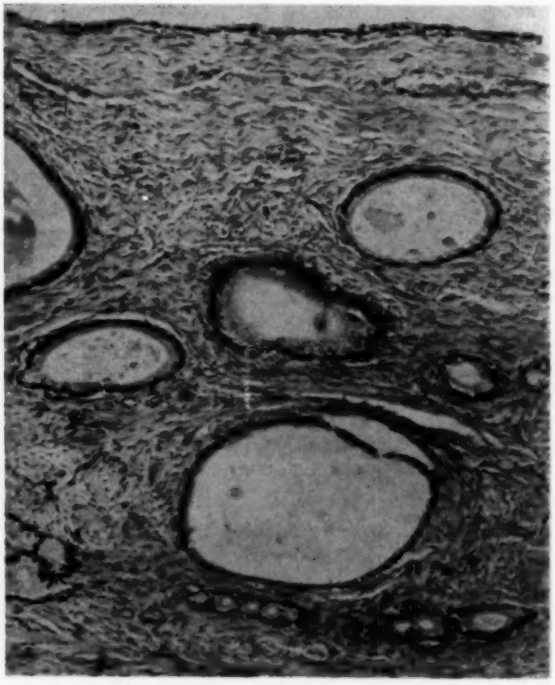

FIG. 3

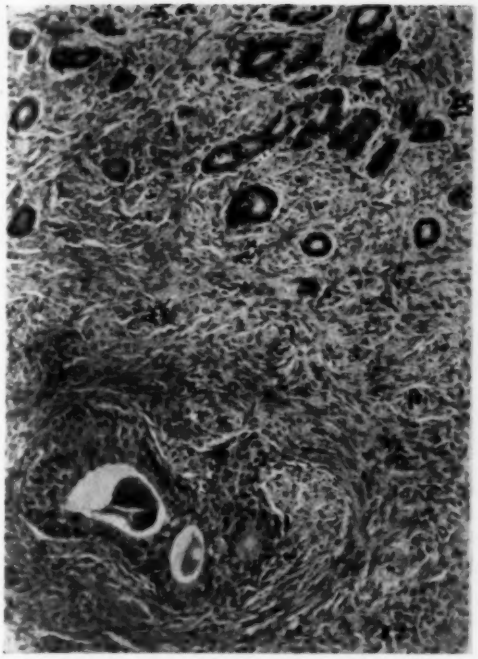

FIG. 4.—Nest of Brenner epithelium surrounded by fibroblastic tissue and glandular elements of gastro-intestinal epithelium. Haematoxylin and eosin × 130.

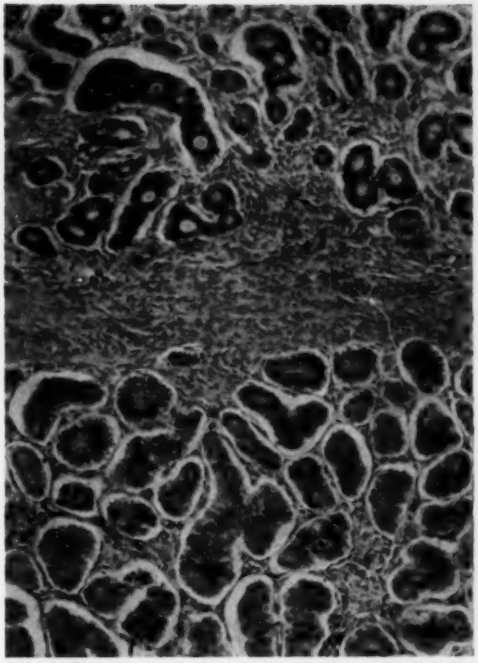

FIG. 5.—Typical compact clumps of carcinoid tissue near acini of gastro-intestinal type. Haematoxylin and eosin. × 130.

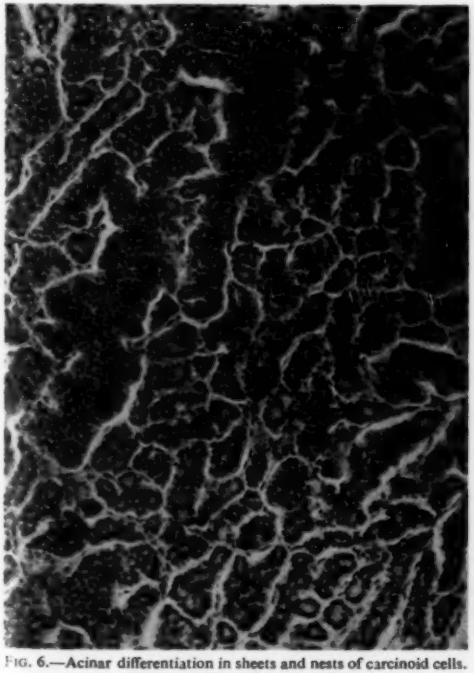

FIG. 6.—Acinar differentiation in sheets and nests of carcinoid cells. Haematoxylin and eosin × 120.

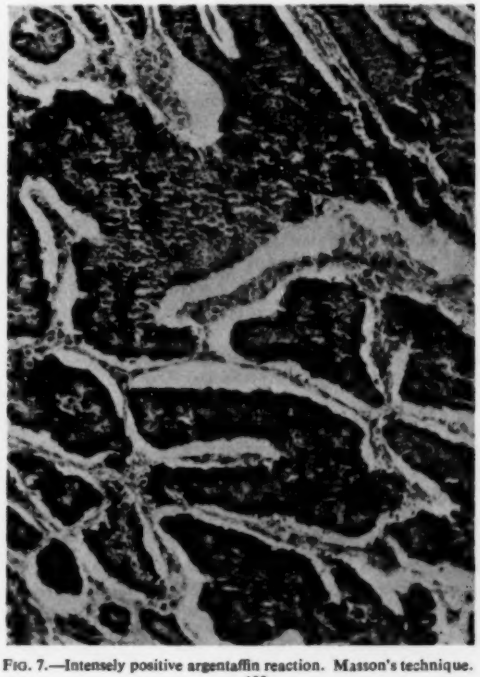

FIG. 7.—Intensely positive argentaffin reaction. Masson's technique. × 130.

The Tumour

Macroscopic Features.—The smoothly and well encapsulated ovarian cyst measured 20 cm. by 18 cm. by 15 cm. and weighed 132 oz. It consisted of one large cavity containing a yellow, turbid, mucinous fluid; occupying a portion of the cyst wall was a distinctly yellow, ovoid, homogeneously solid tumour measuring 10 by 7 by 6 cm. The cyst wall, though thin and stretched, was obviously tough; in the immediate vicinity of the solid mass it was thicker, partly poriferous, and seemingly split into two main layers which encompassed the yellow tumour. The spongy portion consisted of tiny cysts distended by a mucinous fluid, and the inner layer enveloping the solid tumour was intact except in one small area where the yellow neoplastic tissue, having penetrated through this barrier, protruded in warty fashion into the main cavity of the cyst.

Histological Appearances.—The solid tumour exhibited in all areas the characteristic microscopic features of an argentaffin tumour (Figs. 5–7), the carcinoid tissue occurring in compact nests, clusters, and irregular sheets within which tiny acini and pseudo rosettes were elaborated frequently. The cells, especially those peripherally placed in the nests and irregular masses, reacted positively with Schmorl's method; they also reacted positively with the diazo and the Gibbs method for argentaffin granules and yielded a strongly positive argentaffin reaction (Masson) (Fig. 5). The typical silver-reducing and histochemical reactions of the carcinoid cells were obvious and easily elicited in most areas of the tumour; in some parts the reactions were weak and here and there were indifferent and equivocal.

No gastro-intestinal or bronchial structure as such was identified, but in the peripheral borders of the mass there were collections of glandular elements, not unlike atypical gastric epithelium, lined mainly by cuboidal cells. There was, however, no associated smooth muscle, and the acini, in some areas (Fig. 5) separated by fibrous tissue from the more obviously carcinoid tissue, were, in others, intimately related. Such tubular and acinar elements also contained argentophil cells.

The lining of the main cyst either was not recognizable as an epithelial layer or was extremely attenuated and composed of flattened cells. In the poriferous areas near the solid tumour, however, there were small mucinous cysts lined by tall columnar cells with parallel sides and basal nuclei, generally resembling those of pseudomucinous epithelium (Figs. 1–3). Argentaffin cells in the epithelium of these microcysts were extremely scanty (Fig. 2) and very widely dispersed. Although many areas were examined, nowhere could it be established that the carcinoid tissue was in continuity or even in juxtaposition to this pseudomucinous epithelium.

Near the capsule of the cyst and forming part of the solid tumour there was a microscopic nodule of fibroblastic tissue containing nests of typical Brenner epithelium (Fig. 4). The cells in these nests did not reduce silver solutions neither did they react when treated with the diazo and the Gibbs method for argentaffin granules. The nuclear morphology was entirely different from that of the carcinoid cells—many of the nuclei in the clumps of Brenner epithelium exhibiting the characteristic coffee bean appearance. Although a few Brenner nests were cystic none contained a mucinous type of epithelium.

Epidermoid structures, muscle, cartilage, and bone were completely absent from any part of the cyst or solid tumour.

Discussion

The structural features and staining reactions of the solid portion of this predominantly cystic ovarian growth are characteristic of carcinoid tissue. The argentaffin reaction, though fickle and in itself not absolutely specific for carcinoid tumours, nevertheless can be considered conclusive when supported by a battery of histochemical reactions such as those elicited by the diazo, Gibbs, and Schmorl techniques.

Despite the argentaffin nature of the neoplastic tissue, in the absence of evidence obtainable only at necropsy, it might be argued that the growth represents a secondary, not a primary, carcinoid tumour of the ovary. Unlike the majority of instances of secondary ovarian carcinoid tumours, however, the growth in this case was a unilateral gonadal tumour; in addition, the origin of the argentaffinoma from a gastro-intestinal type of epithelium included in the wall of this cystic ovarian teratoma was clearly demonstrable on microscopy.

In the reported instances of primary ovarian carcinoid tumours the growths have arisen from either gastro-intestinal or respiratory epithelium in dermoid cysts. The case described in this communication appears to be the first occasion when such a carcinoid tumour has been observed to originate in association with a pseudomucinous cyst, particularly one which also contained within its wall a Brenner tumour of microscopical

dimensions. The full potentialities of the ovarian stroma remain largely undisclosed so that the histogenetic implications of the association of pseudomucinous epithelium with teratoid elements is difficult to evaluate. Nevertheless the possibility that a pseudomucinous cystadenoma may arise as an exaggerated development of the endodermal component of a teratoma must be admitted even though conclusive evidence of such a mode of origin is not yet available. Although the teratoid theory of genesis of pseudomucinous epithelium is considered tenable but unproved, it is not suggested that in this case such an epithelium, together with the gastro-intestinal epithelium, the argentaffinomatous tissue, and the very small Brenner tumour, are all elements of a single teratomatous growth. No direct continuity between the carcinoid and gastro-intestinal elements, on the one hand, and the pseudomucinous lining on the other hand could be established, and it is likely that, whereas the gastro-intestinal epithelium and the argentaffinoma arising from it are teratomatous in nature, the accompanying pseudomucinous cystadenoma is of independent origin.

The "hot flushes" experienced by the patient became progressively less marked after the removal of the ovarian teratoma; yet they are of considerable interest because of their association with a large argentaffinoma. Although no direct proof exists the possibility remains, nevertheless, that this symptom might have been accompanied by a pre-operative, high urinary output of 5-hydroxyindoleacetic acid. Perhaps, on account of its large bulk, the teratomatous argentaffinoma was capable of secreting humoral agent in sufficient quantities to cause these episodic flushes, so characteristically exhibited in the carcinoid syndrome.

Primary argentaffinomatous tumours of the intestine, by the time the carcinoid syndrome is established, usually have produced intrahepatic secondary deposits. In the case of the primary ovarian tumour discussed in this communication, post-operative tests for 5-hydroxyindoleacetic acid were repeatedly negative, suggesting that the growth, before its removal, probably had not produced many, if any, gross metastases.

Summary

An argentaffinoma arising primarily in an ovarian teratoma associated with a pseudomucinous cyst is described. It is believed the case represents the eighth instance in which a carcinoid growth has been observed to arise primarily in the ovary.

For his interest and for allowing access to the clinical notes we are very grateful to Sir Arthur Gemmell, M.C., T.D., M.A., M.D., F.R.C.S.Ed., F.R.C.O.G.

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TECHNICAL METHODS

A Technique for Obtaining Undistorted Specimens of the Central Nervous System

K. M. LAURENCE* AND DEREK MARTIN

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(RECEIVED FOR PUBLICATION AUGUST 20, 1958)

The conventional methods used for the removal of the central nervous system may lead to distortion of the brain, and it is difficult to obtain an intact specimen in certain pathological conditions without employing some special technique. This is particularly true in cases of hydrocephalus where the "cerebral cortex" is frequently so thin that the brain collapses as soon as the skull cap is removed.

A special technique for the removal of the brain and spinal cord *in situ* within the bony cage was described by Martin (1952). There was one serious disadvantage in this method, as the cranial cavity had to be opened, with the possibility of distortion of its contents. The present method obviates such a possibility and is an improvement on that originally described.

Method

FIG. 1.—After disarticulation, the spinal column is dissected from the attached structures as far as the base of the skull.

Removal of Specimen.—The viscera are removed in the customary way to expose the entire length of the vertebral column. The sacro-iliac joints are disarticulated with a sharp cartilage knife or saw, and the costo-vertebral ligaments are cut. The vertebral column is then dissected away from skin and other structures as far as the base of the skull (Fig. 1). If a spina bifida cystica is present it is advisable to make incisions from behind through the skin around the lesion before the above procedures and remove the latter together with the vertebral column.

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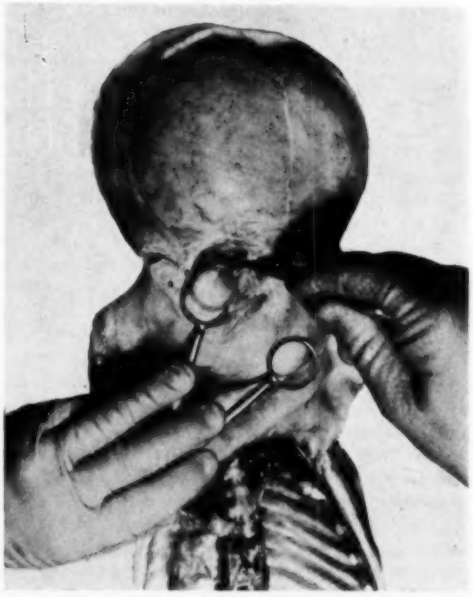

FIG. 2.—This shows the mobilization of the eye from the orbit.

With the body in the prone position, taking care to protect the facies from pressure, an incision is made from the vertex (anterior fontanelle) to the nape of the neck. The scalp is reflected from the calvarium as far as the ramus of the mandible, the attachments of the pinnae are severed, and the temporo-mandibular joints disarticulated.

The body is then placed in the supine position and the scalp is retracted over the face as far as the superior orbital margins and the bridge of the nose. Care must be taken to avoid cuts in the skin because of difficulties with subsequent reconstruction. The skin is then dissected from the bridge and sides of the nose, and the orbital septae are cut to expose the peri-orbital fat. The round handle of a pair of scissors is inserted behind the eyeball, which is retracted forward, and its muscles and nerves are severed (Fig. 2). The eyeball thus remains attached to the skin by its conjunctiva and subconjunctival tissues only. The scalp and attached eyes are deflected forwards and an oblique saw-cut is made across the bridge of the nose and zygoma to the pharynx (Fig. 3). The skull and the vertebral column are finally freed from the rest of the body by cutting the remaining fascial strands and muscles.

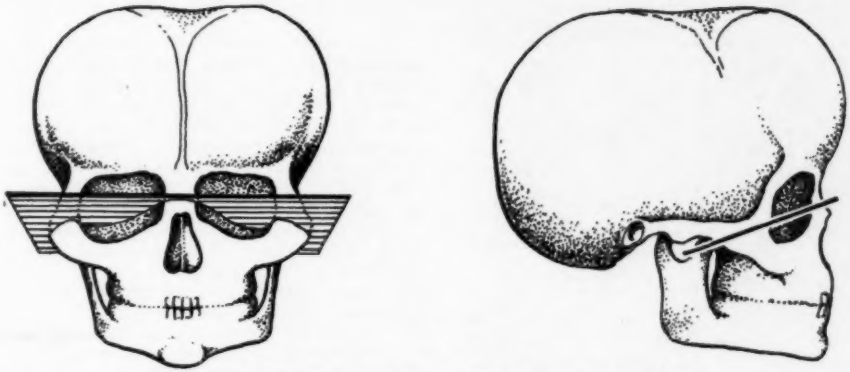

FIG. 3.—Diagrams to illustrate the plane of the saw cut through the facial bones.

Altogether these procedures should not take longer than half an hour in a child, although in an adult more time may be required.

Fixation.—Fixation is critical and necessitates some care. The best results are obtained when the carotid arteries are perfused with saline solution and then 10% formol saline before necropsy. Unfortunately, this technique may produce wrinkling and shrinkage of the facial skin, which makes the subsequent reconstruction difficult. This method, therefore, is only applicable to selected cases.

More generally, fixation is achieved after removal of the specimen by needling the ventricular system either through the anterior fontanelle or a burr hole and replacing the bulk of the cerebrospinal fluid with 15% formol saline. The needle is then partly withdrawn and a small quantity of the fixative is instilled in the subdural space. Fixative is also introduced into the lumbar theca in much the same way as a lumbar puncture is performed. The whole specimen is then placed in a bath of 10% formol saline in, as nearly as possible, its correct anatomical position, and fixation is allowed for at least one week, but preferably for three.

Reconstruction.—The reconstruction of the body is carried out in the usual way. The vertebral column is replaced by a measured wooden prop, and a representation of the skull is modelled in moist lint and cotton wool. After suitable packing, the abdomen and neck incisions are sewn up. The head is best fashioned into the correct shape upon the measured prop before placing the latter in position, using the specimen as a guide to the exact shape required. Two depressions are made for the orbits, into which the eyes can be inserted. The scalp is then partially stitched up and moulding is completed with pledgets of moist cotton wool. Head measurements made before the necropsy may be of assistance. With the above technique, especially in cases of hydrocephalus, subsequent appearances may indeed represent a cosmetic improvement on the original.

Dissection of the Specimen.—The precise method adopted for the dissection is obviously largely dictated by the suspected lesion and the structures to be studied primarily. In practice, the most satisfactory method is to make a mid-line saw-cut through the skull, spines, and vertebral bodies, without damaging the dura. The hemisection of the vertebrae, starting at the coccyx, is completed with a cartilage knife.

After severing the spinal nerve roots as closely as possible to their entry into the intervertebral foraminae, one half of the column is removed, leaving the dura intact. One half of the calvarium is then removed piecemeal with bone forceps and saw, without damaging the underlying dura (Fig. 4). The latter is then stripped off the exposed portion of the cord and brain; the tentorium, transverse sinus, falx cerebri, and the superior sagittal sinus are left *in situ*. The exposed surfaces of the brain and cord can then be examined in detail. Further dissections are made, or the brain and cord removed *in toto*, depending on the nature of the findings. For general purposes of demonstration, the method of choice is a mid-sagittal section achieved by carefully separating the cerebral hemispheres and then cutting down the cerebrospinal axis (Fig. 5).

FIG. 4.—The specimen after removal of half the bony cage; the dura remains intact.

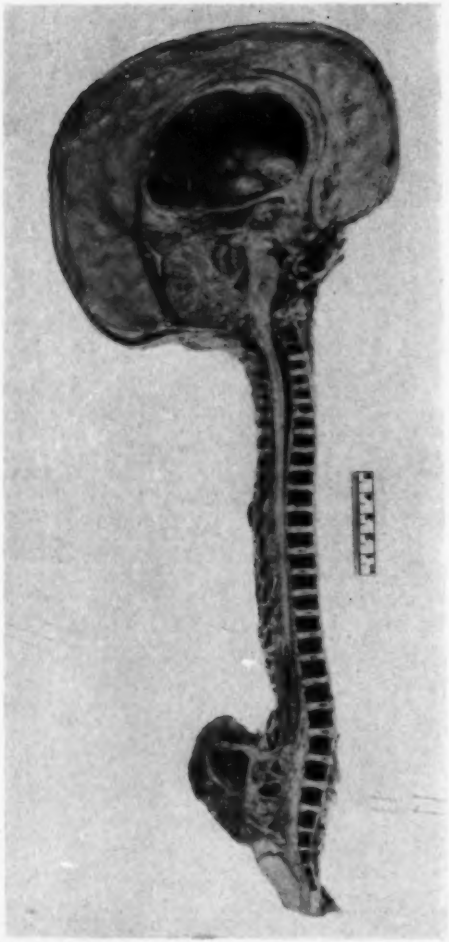

FIG. 3.—A complete hemisection of the central nervous system showing hydrocephalus, small Arnold-Chiari malformation, and a sacral myelocele.

Discussion

This method permits the preservation of precise anatomical relationships, such as those of the basal cisterns, without the possibility of distortion. It is also ideal for obtaining specimens for demonstration and research. If an immediate opinion on the central nervous system is required, the technique is obviously not practical, nor is it recommended for routine use. This method is, however, ideal for the investigation of such problems as hydrocephalus. A brain with attenuated cerebral tissue may be examined, when fixed, without danger of distortion after removal from the cranium. This is probably the only way whereby the basal cisterns may be studied post mortem. Minor degrees of tentorial herniation, otherwise often missed (Laurence, 1957), can also be easily recognized. The Arnold-Chiari malformation is preserved in its correct relationship to the surrounding bony structures, and even minor deformities can be demonstrated. The study of other malformations, including cranial spina bifida, agenesis of the corpus callosum, and porencephaly, and also cerebral tumours is greatly facilitated. By this method the brain can also be studied after recent extensive intracranial operations, which may make examination by conventional procedures difficult.

Summary

A method of removing the central nervous system within the bony cage is described. This enables undistorted specimens to be examined in their correct anatomical relationships, and is an ideal technique for teaching and research. Some special applications of the method are suggested.

We wish to thank Dr. M. Bodian for his advice and permission to publish and Mr. D. Lyth for Fig. 3; One of us (K. M. L.) is indebted to the Research Committee of The Hospital for Sick Children, Great Ormond Street, for support.

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Techniques Used in the Routine Examination of Specimens for the Specific Serological Types of *Escherichia coli*

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From the Children's Hospital, Birmingham

(RECEIVED FOR PUBLICATION JULY 27, 1958)

In this routine laboratory every "coliform" colony from the cultures of any specimen, except colonies on desoxycholate citrate plates, is examined by the following methods to identify specific serotypes of the *Escherichia coli*. The techniques employed are designed to provide reliable results in the shortest possible time.

Rapid Tube Agglutination Using a Centrifuge

Each serotype, from a new patient or specimen, which is identified by slide agglutination must have its identity confirmed by a tube agglutination test carried out to the titre of the specific antiserum (Taylor, 1956). It is the practice here to perform slide agglutinations between 9 and 10.30 in the morning.

A portion of a colony which has given a positive slide agglutination is subcultured into a tube of warm infusion broth, a batch of which is always kept in the 37° C. incubator. After four hours' incubation there is sufficient growth available for confirmatory tube agglutinations to be carried out. Some of the broth culture is used to set up the confirmatory tests that the laboratory employs for the identification of the members of the *Enterobacteriaceae*, and a drop is spread on a MacConkey plate, which is incubated, and single colonies examined the next day to ensure that only one serotype is present. The remainder of the broth culture is employed for the confirmatory tube agglutination tests. One portion is boiled for 15 minutes and is then centrifuged at 3,000 revolutions per minute for five minutes. The deposited organisms are re-suspended in a suitable quantity of saline to give a density of about 250 by 10^6 organisms per millilitre and they are used as the O antigen for agglutinations in round-bottomed 2 in. by $\frac{1}{4}$ in. test tubes. The tubes are placed in a water-bath at 50° C. A control tube, in which the antiserum is replaced by normal saline, must be incubated at the same time. After 15 minutes' incubation the tubes are numbered, and then centrifuged for one or two minutes at about 1,000 revolutions per minute. After centrifuging the tubes are placed in a rack and each is rotated rapidly within the rack by a series of stroking movements made with the index finger on the side of the tube. The mixing is continued until the organisms in the control tube have made an even suspension: if specific agglutination has taken place the aggregates can then be seen as coarse clumps. It sometimes helps to dislodge the deposited organisms if the tube is gently tilted. The supernatant fluid runs down the tube and forms a new meniscus, behind which there is an air bubble, which runs up the inverted tube and displaces the clumped organisms from whence they are deposited on the rounded bottom (Fleming, 1928).

Braun, Seeliger, and Wagner (1954) used a centrifuge technique for the demonstration of O agglutinins in the sera of babies who had had *E. coli* gastro-enteritis. They employed this method because conventional incubation of the agglutination reaction failed to show any antibodies in the same sera.

It is really unnecessary to examine each strain for both K and O antigens, but if both are required some of the broth culture is centrifuged and the centrifuged deposit killed by the addition of three drops of 1:1,000 mercuric iodide in saline: this suspension is employed as the antigen for a K agglutination test. The K agglutinations are made, as is usual, in 4 in. by $\frac{1}{2}$ in. test tubes. The tubes are incubated for 15 minutes at 37° C., then centrifuged for one minute at 1,000 revolutions per minute, and after this the bottom of each tube is flicked in the routine that is used to demonstrate the presence of a K agglutination (Kauffmann, 1951).

Using these techniques it is possible to provide the results of the confirmatory tube agglutinations in the late afternoon the day after a specimen has been received, but, as the faeces of a baby with enteritis due to one of these specific serotypes contain vast numbers of that serotype in a nearly pure culture (Rogers, 1951 ; Thomson, 1955), it is possible to make a saline suspension from the thickly inoculated portion of a plate and to provide a tentative diagnosis four hours after planting a specimen. A rapid diagnosis of a specific serotype infection permits chemoprophylaxis to be given early both to the affected baby and to any other infants in the ward.

When a great number of culture plates have to be examined, rapid screening can be effected if each culture is examined by slide agglutination of a suspension made from the confluent part of the growth. For two years investigations have been begun with this technique, a note has been made of the results, and then at least four individual colonies have been tested from that portion of the plate on which differentiation has provided separate discrete colonies. It is usual for this preliminary screening to demonstrate a specific serotype if any is present on the plate.

Saline Pencil Technique

In her description of the preliminary slide agglutinations, Taylor (1956) briefly described a technique using ordinary clean 3 in. by 1 in. microscope slides on which colonies are suspended in drops of normal physiological saline. This saline is dispensed from a pencil and a thin wire loop is used to remove a small portion of the colony.

A reservoir type Pasteur pipette is cut off squarely near to where the actual pipette is formed from the main butt, and this square-cut opening closed by melting the glass in a flame until it forms a strong thickened glass end with a pinpoint-sized hole in the centre. As it is narrowed by the heating the size of the aperture can be checked with a hand-lens, or, when the pipette is cool, by filling it with water and seeing how much comes out, when the pipette is touched on to a glass slide. If the hole is too large and fluid comes out too quickly, the water is shaken out and the glass re-melted to make the hole smaller. Before the saline pencil is put into use, the end opposite to that with the pinhole opening is heated in the flame until it is soft enough to be bent, so that the pipette becomes eccentric: this prevents the pencil from rolling when it is dropped back or placed on the bench.

When it is used the pencil is filled with 0.85% saline and the thickened end is used to tap a slide, on which it then leaves a small drop of saline.

The small wire loop is of 33 S.W.G. (0.01 in. in diameter) michrome or platinum wire made into a 2 mm. loop. This thin wire is used because it cools very quickly after it has been sterilized and, also, its small size allows several portions of any colony to be removed.

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BOOK REVIEWS

Chromatographic Techniques. Clinical and Biochemical Applications. By Ivor Smith; Foreword by C. E. Dent. (Pp. xiii+309; illustrated. 45s.) London: Heinemann. 1958.

Chromatography has not been altogether neglected in the ever-increasing flow of new publications in biochemistry, but there has been a real need for a good practical book on filter paper chromatography to which the clinical biochemist can refer for guidance. Dr. Smith, with the assistance of several colleagues, has successfully remedied this deficiency and presents here a mine of information for the identification of many substances of medical importance. Much of it is new and hitherto unpublished material.

The first chapters are given to general principles of technique, and the author describes a simple "universal" apparatus for ascending and descending chromatography using 10 in. square papers. There is a good account of preparatory methods including the desalting techniques, viz., electrolytic desalting, ion exchange separations, and organic solvent extraction. The electrolytic desalter perfected by Dr. Smith and his associates is discussed in detail. Those who do not possess a desalter will find the alternative methods helpful, but the author has not applied them so much to body fluids so that a certain amount of preliminary testing is still required to secure optimum conditions before putting these into routine use.

Following chapters deal specifically with the analysis of amino-acids, indoles, imidazoles, guanidines, purines and pyramidines, sugars, ketoacids, phenols, barbiturates, steroids, etc. In each case clear instructions are given regarding the choice of solvent and location agents, a general procedure is laid down, location maps are usually provided, and the clinical applications discussed. The sections on amino-acids, indoles, and steroids are particularly helpful. Some of the other sections are of necessity short, and their relation to routine clinical biochemistry is at the moment limited.

If there is any weakness in the book, it is in the clinical notes, which contain some unusual statements, e.g., glycosuria is suggested as a possible guide to treatment and progress of renal tubular necrosis, and a distinction is drawn between cystinosis and the Fanconi syndrome. Crystals of cystine are said to have a "characteristic octagonal shape."

These sections will require revision in any future edition, but the real value of the book lies in its wealth of technical detail not readily available elsewhere, and for this reason it is likely to become a standard manual in its field.

W. H. R. Auld.

Practical Clinical Biochemistry, 2nd ed. By H. Varley. (Pp. viii + 635; 78 figures. 42s.) London: Heinemann. 1958.

The second edition of this work remains an excellent collection of methods. Some of the new methods included are very useful: this is particularly so for such tests as the qualitative test for increased total 5-hydroxyindole excretion. The new chapter on hormones is good. Although the choice of the particular method may still be a problem (an *embarras de richesses*) this edition is a little more helpful than the earlier one. A valuable addition is a section on the control of accuracy. It is to be regretted that not only has the opportunity been neglected to eliminate errors in the spelling of names which occurred in the first edition (Wootton on p. 413 and Dyke on p. 600 are both misspelt in both editions), but names occurring for the first time in this edition are misspelt (e.g., McMillan on p. 530). A third edition may confidently be expected; it is to be hoped that the author will take the opportunity to eliminate some of the methods, such as the open-circuit method of determining the basal metabolic rate and some of the seven blood sugar methods; methods retained could well be expanded to include a good deal more discussion of the difficulties that laboratories encounter when using them. In the present spate of books describing methods in clinical biochemistry, "Varley" is one of the few worth having. That the price has remained unchanged from one edition to the next is noteworthy.

ARTHUR JORDAN.

Clinical Chemistry in Practical Medicine, 5th ed. By C. P. Stewart and D. M. Dunlop. (Pp. vii + 342; 30 figures. 27s. 6d.) Edinburgh and London: E. and S. Livingstone. 1958.

This book has now reached its 28th year and fifth edition, which in itself signifies its usefulness to generations of students and graduates. It has been revised and partly rewritten, and is now up-to-date enough to include a section on the transaminases though as yet no mention of haptoglobins. It covers all the needs of students and of most clinicians.

The average standard is high but rather uneven. The chapters on plasma proteins, carbohydrate metabolism, gastric function, and thyroid function impress one as particularly valuable. The chapter on acidosis and alkalosis, however, is likely to confuse many medical readers; the authors use the nomenclature by which the term "base" is defined as a substance which tends to accept hydrogen ions, and therefore includes anions such as HPO_4 and HCO_3 (which are often called "acid radicals") and excludes cations such as Na and K (which are included in the physiologist's expressions "base conservation" and "fixed base"). As the term "base" is so firmly entrenched in medicine in its old sense, it would be preferable to interpret acid-base balance in terms of acid and alkali, and anion and cation.

There are a few mistakes, such as the definition of congenital porphyria as a variety of porphyria cutanea tarda which does not appear until adult life; and some of the interpretations will not be generally accepted, e.g., that for serum amylase "a figure for over 300 Somogyi units is clearly indicative of acute pancreatitis." Nevertheless this book, well set out, easy to read, and moderately priced, remains in general a very useful interpretation of chemical pathology to the clinician.

G. KEMP MCGOWAN.

Principles of Biological Microtechnique. By J. R. Baker. (Pp. 368; 8 half-tone plates, 14 text figures. 45s.) London: Methuen. 1958.

Essentially this book is an elaboration of the author's views recorded in former years in succeeding editions of *Cytological Technique*. The two main sections of the book deal with fixation and dyeing respectively. In the first the author considers the effects of a wide variety of fixatives on various tissue components, and in the second he considers the structure of dyes and the whole mechanism of staining. Succeeding chapters deal with metachromasy, blood dyes, and vital staining.

The book is not to be regarded as a handbook but as a critical and stimulating review. Histologically and histochemically it is strongly orientated towards zoological techniques, and the pathologist may find his favourite methods are wholly ignored. Nevertheless, he will do well to read it.

A. G. EVERSON PEARSE.

Absorption Spectrophotometry. By G. F. Lothian. (Pp. viii + 246; 68 figures. 52s.) London: Hilger and Watts. 1958.

It is nearly 10 years since the first edition of this book was published and this second edition has been almost completely rewritten. As before, the book is divided into three parts: the first on principles of spectrophotometric measurement; the second, the application of these measurements for chemical and analytical purposes; the third, an up-to-date section which deals with the general design of spectrophotometers of various types. This section is illustrated by a great number of references, plans, and pictures of available commercial instruments both British and foreign.

It will be readily seen that a very large field is covered by Mr. Lothian and he necessarily does not go very deeply into detail. Nevertheless one has the impression of a very even treatment of this extensive field, and this book can be recommended with confidence to any person who requires a knowledge of spectrophotometry as an analytical tool. There is, however, one reservation; during recent years there has been an enormous increase in the use of spectrophotometry by scientists in many fields. The specificity and sensitivity of the methods, together with the success of instrument makers in producing robust instruments at a relatively low cost, has brought spectrophotometry into many laboratories which are

not staffed by people with an advanced formal physical education. As a result there is a growing body of people who use sensitive spectrophotometers without any clear understanding of the physics of their measurements, and I am not convinced that the presentation here is ideally suited for such people. Moreover, there is a flavour of the physicist about the derivations of the various equations. For example, the chapter on "laws of absorption" opens with a statement of Lambert's law in the form of a simple differential equation, which is integrated to give the familiar relationship between incident and transmitted light intensity. This is an elegant and economical treatment but is not, to my mind, the right approach for teaching the subject to (say) a zoologist using spectrophotometry for a biochemical estimation. Nevertheless, providing the reader is not frightened by the notation of elementary calculus, this book is readable and can be recommended.

I. D. P. WOOTTON.

The Determination of the ABO and Rh(D) Blood Groups for Transfusion. Medical Research Council Memorandum No. 36. (Pp. vi+46; 7 tables, 1 figure. 3s. 6d.) London: H.M.S.O. 1958.

As the authors state in their preface, blood transfusion has achieved such a prominent place in modern medicine that the present memorandum is, of necessity, much more than a revision of War Memorandum No. 9, 1943.

The working party set up by the Medical Research Council, consisting as it does of practical blood group serologists in daily touch with the complexities and dangers of grouping and cross-matching, concentrates on those techniques which have stood the test of time and stresses the need for these important investigations to be carried out only by those with special training.

It rightly emphasizes that in no other sphere of pathological investigation is an error fraught with such danger to the patient.

The whole memorandum is admirably set out, and, in particular, section VII, dealing with direct matching tests, is strongly recommended for study by all undertaking blood transfusion work.

The rapid direct matching test for use in emergency will be found particularly valuable in the laboratories of those hospitals dealing with accident cases.

R. A. ZEITLIN.

The Cytology of Effusions in the Pleural, Pericardial and Peritoneal Cavities. By A. I. Spriggs. (Pp. 71; 40 figures, 5 colour plates. 42s.) London: Heinemann. 1957.

Textbooks of cytology with their photographs and coloured plates often have a considerable aesthetic appeal. That written by Dr. Spriggs is no exception. Added virtues are a critical yet lucid text, plenty of references, and the advocacy of Romanowsky stains. As an adjunct to standard textbooks and atlases of haematology this book can be recommended; but one may, perhaps, jib slightly at the price.

HUMPHREY KAY.

Coroner's Practice. By Gavin Thurston. (Pp. 180. 21s.). London: Butterworth. 1958.

In this short and handy publication the functions and duties of a coroner are clearly outlined. Its aim is to provide useful information for those beginning a career as coroner, and Dr. Thurston is to be congratulated on the balance of legal and medical information provided. This, together with the clarity of style, makes it particularly valuable for senior and junior persons who lack either legal or medical qualifications.

The chapter on accidental death is timely in view of the number of fatal accidents on the road or in industry, and the section on industrial diseases most useful. Some basic pathology is adequately dealt with under post-mortem examination, though it is hoped that in future editions of the work a little more space will be devoted to the pathological effects of coronary thrombosis. In many instances a present coronary thrombosis is accompanied by scarring, indicative of previous similar thromboses.

Dr. Thurston's work is certain to be well received and will be a valuable asset to those engaged in forensic work, and comes appropriately at a time when forensic pathology is being recognized as an important specialized branch of the parent subject.

G. J. CUNNINGHAM.

Corrections

In the paper by I. G. Graber and S. Sevitt (*J. clin. Path.*, **12**, 25) there are two errors. (1) On page 27, the footnote to Table II, after fraction of filtered sodium excreted, read "A=normal or decreased from admission. B=early increase with fall to normal or decreased values. *Anuria after 24 hours." (2) In the legend to Fig. 8, for "on the right" read "on the left" and for "on the left" read "on the right."

In the paper by E. J. King and K. A. Jegatheesan (*J. clin. Path.*, **12**, 85), under the heading "Reagents," the second paragraph should read as follows:

Substrate (0.01 M-Disodium Phenyl Phosphate).—For the substrate, dissolve 2.18 g. disodium phenyl phosphate in 1 litre of freshly boiled and cooled distilled water and preserve with a few drops of chloroform in the refrigerator.

J. clin. Path. (1959), **12**, 195.

ACIDOSIS AND ALKALOSIS: A MODERN VIEW

BY

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With respect to the interrelations of acids and bases in mammalian organisms the maintenance of the hydrogen ion concentration within a rather narrow range appears to be of paramount importance. Secondary, or ancillary to this, but of nearly equal importance, is the mechanism by which future or potential disturbances of the acid : base equilibrium may be rectified. Remarkable progress has been made in unravelling the exceedingly complex processes which are involved, but until recently the work has undoubtedly been hampered by the use of concepts concerning the nature of acids and bases which, though in accord with our knowledge at the time they were introduced, have since been superseded by the simpler ideas now generally accepted by physical chemists. We believe that the application to biochemistry of these ideas, and the terminology based upon them, is capable of clarifying the subject of hydrogen ion regulation and, consequently, of acidosis and alkalosis. In particular, it emphasizes the role of the bicarbonate ion, it facilitates understanding of the renal mechanism, and it affords an explanation of the secondary but important role of metallic cations such as sodium and potassium which are neither bases nor acids, and of anions such as chloride which are not effective bases. This paper is an attempt, brief and necessarily incomplete, to base a description of acidosis and alkalosis upon chemically acceptable definitions of acids and bases.

ACIDS AND BASES

An "acid" is defined, according to the theory independently enunciated by Lowry and Brönsted, as a substance which, in solution, tends to liberate hydrogen ions (protons); these hydrogen ions may remain as such, or may combine with the solvent forming, e.g., hydroxonium ions, H_3O^+, or may combine with negatively charged ions. A "base" is similarly defined as a substance which tends to accept hydrogen ions.

Since the dissociation of an acid is reversible, the negatively charged anion which is liberated along with the hydrogen ion is a base, and the older term "acid radicle" for such an anion is misleading.

$$\underset{\text{(Acid)}}{H^+ \text{ donor}} \rightleftharpoons H^+ + \underset{\text{(Base)}}{H^+ \text{ acceptor}}$$

Acids, though frequently undissociated molecules, are not necessarily so, and bases, though the class includes all anions, may be molecules. Thus the cation NH_4^+ liberated from ammonium salts is an acid because it is capable of producing hydrogen ions according to the equation:

$$NH_4^+ \rightleftharpoons H^+ + NH_3$$

From this equation it is evident that the ammonia molecule NH_3 is a base. Similarly the anion $HPO_4^=$ is a base because it can accept H^+ to form $H_2PO_4^-$ which can accept more H^+ to form the acid molecule H_3PO_4. But the anions $H_2PO_4^-$ and $HPO_4^=$ are also acids because they can lose H^+ to form, ultimately, the base $PO_4^{\equiv}$; they are "amphoteric."

These definitions leave undisturbed many of the familiar concepts. Thus the acidity or alkalinity of a solution is still expressed in terms of hydrogen ion concentration or of *p*H, the logarithm of its reciprocal.

They emphasize the fact that the acidity of a solution does not depend solely on the concentration of acid present but also on the degree of dissociation. A decimolar solution of hydrochloric acid (almost completely dissociated) has a much greater acidity than a decimolar solution of acetic acid only about 2% dissociated.

They permit us to deduce from the equation: $HX \rightleftharpoons H^+ + X^-$ the standard formula: $pH = pK + \log_{10}\frac{[Base]}{[Acid]}$.* They avoid the confusion which arises from the common misconception that anions are acids and that metallic cations are bases. This does not mean that cations such as

*The symbol [X] represents the concentration of X expressed in g. ions or g. molecules (as the case may be) per litre of solution.

Na^+ and K^+ are of no importance, but it does make it clear that they are not primary regulators of the acid : base equilibrium. Their importance—apart from any special functions of their own—is that electrical neutrality must always be maintained ; anions and cations must always be present in equal concentrations, and a "cation-anion imbalance," to which some writers refer, can never, in fact, occur.

The "strength" of an acid is measured by its tendency to donate hydrogen ions or, in other words, by its degree of dissociation ; the freely dissociated mineral acids (HCl, etc.) are strong acids ; slightly dissociated acids such as carbonic acid are weak. Similarly a strong base has a great affinity for hydrogen ions whereas a weak base is a poor acceptor. The important corollary of this latter fact is that the anion of a strong acid is a weak base, e.g., Cl^-, and the anion of a weak acid is a strong base, e.g., HCO_3^-, OH^-. The physiologically important acids can, on this basis, be arranged in decreasing order of strength, and if this is done as in Table I the corresponding bases are automatically arranged in increasing order of strength.

TABLE I

ACIDS IN ORDER OF DECREASING AND BASES IN ORDER OF INCREASING STRENGTHS

Acid		Base
HCl	⇄	$H^+ + Cl^-$
$H_2PO_4^-$	⇄	$H^+ + HPO_4^{--}$
H_2CO_3	⇄	$H^+ + HCO_3^-$
HPr*	⇄	$H^+ + Pr^-$
NH_4^+	⇄	$H^+ + NH_3$
H_2O	⇄	$H^+ + OH^-$

* Under physiological conditions nearly all proteins are actually ions which are, like $H_2PO_4^-$, still donors of H^+. Some of these proteins are actually rather stronger acids than H_2CO_3, some weaker.

BUFFERS

A "buffer system," which minimizes *p*H changes on addition of acid or base, consists of a solution containing a weak acid together with one of its soluble salts. The acid, being weak, is slightly ionized ; the soluble salt, however, is ionized to a large extent. The mixture thus provides a reservoir of base (anions) which can combine with added H^+, i.e., "neutralize" added acid, and a reservoir of acid (undissociated acid molecules) which can donate hydrogen ions to neutralize added base. Thus a mixed solution of carbonic acid and sodium bicarbonate acts as a buffer system. It contains undissociated carbonic acid (a weak acid), a small amount of hydrogen ion, sodium ion, and bicarbonate ion (a strong base) derived from nearly all the sodium bicarbonate of which only a little remains undissociated. Its actual *p*H depends as usual on the ratio between the concentrations of base and acid.

On addition of hydrochloric acid, the H^+ ions of this acid nearly all combine with the bicarbonate ions to produce feebly dissociated carbonic acid so that the actual increase in acidity is only a small fraction of that which would be caused by the hydrochloric acid in water alone. Every bicarbonate ion removed is replaced by a chloride ion so that the total anion concentration is maintained unaltered and, as must necessarily be the case, cation-anion balance is maintained. The removal of H^+ can only proceed so long as bicarbonate ions remain ; in other words the neutralizing capacity (= buffer power) for acids is quantitatively limited by the concentration of the base.*

When a base is added to this buffer system a similar chemical action occurs and again the concentration of hydrogen ions is stabilized. Sodium hydroxide, for example, which is almost completely ionized in solution with liberation of the very strong base, hydroxyl ion, reacts with the carbonic acid forming water. More precisely, the OH^- combines with the free H^+ to produce water, and, the equilibrium, $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, being disturbed, more carbonic acid dissociates, the process continuing until either the acid or the OH^- is completely used up. The effect is that, so long as the buffer system remains unexhausted, some carbonic acid has been converted to bicarbonate ions (enough to remove the hydroxyl ions as water) and the remainder is, as usual, slightly ionized so that the concentration of hydrogen ions is very little altered.

It is clear that in a solution containing several buffer systems they must be, and must always remain, in mutual equilibrium. If, therefore, the ratio of acid to base is known for any one of the systems the *p*H of the mixture is known. Since, in plasma, the bicarbonate-carbonic acid buffer is by far the most important quantitatively and also the easiest to measure, the total CO_2 content of plasma which is the sum of the $[HCO_3^-]$ and the relatively small $[H_2CO_3 + CO_2]$ is commonly taken to represent the total buffering capacity, and though this is not precisely true under all circumstances it is a good approximation for ordinary purposes.

*The buffering capacity with respect to acid is evidently the "available base reserve." It has often been termed the "alkali reserve," but this term is to be avoided since an alkali is really a soluble hydroxide.

THE *p*H-STABILIZING MECHANISMS OF BLOOD AND TISSUES

Haemoglobin has a special place in the *p*H-stabilizing mechanisms of blood because (*a*) haemoglobin and oxyhaemoglobin have different iso-electric points and different ionization constants so that at the *p*H of blood the liberation of oxygen from one gram molecule of oxyhaemoglobin involves the absorption of about 0.7 g. of hydrogen ion (and the release of an equivalent amount of potassium ion in exchange). The hydrogen ion is provided mainly by carbonic acid but could come from any acid which penetrates the red cells. (*b*) Haemoglobin, more easily than other proteins, reacts reversibly with carbonic acid to form a still weaker carbamino-acid; this specific contribution to the disposal of carbonic acid involves an increasing accumulation of carbamino-haemoglobin as the blood passes through the capillaries and a sharp reversal when, in the lungs, the equilibrium is disturbed, carbon dioxide being excreted, carbonic acid converted to carbon dioxide under the influence of carbonic anhydrase, and, consequently, carbamino-haemoglobin decomposed.

The existence of these two mechanisms, backed by the very efficient excretion of CO_2 in the lungs, ensures that, so long as the respiratory exchange is normal, extra metabolic production of CO_2 does not result in a disturbance of the acid-base ratio.

The buffers which are effective over the *p*H range of the blood and tissues consist of three systems: bicarbonate-carbonic acid, protein anion-protein, and monohydrogen phosphate-dihydrogen phosphate. Quantitatively, the bicarbonate-carbonic acid system is the most important in the plasma, but is much less so in the cells. These buffers must, for a given fluid, be in mutual equilibrium, and are in electrical neutrality by virtue of the cations, mainly sodium in the plasma and extracellular fluid, mainly potassium in the intracellular fluid. Since, however, the cell membranes are selectively permeable and transfer of ions into and out of the cells is partly governed by this and by the Donnan equilibrium, it is possible at times for the intracellular and extracellular fluids to get "out of step." There may, for example, be an abnormal diminution in the cell buffer capacity not immediately reflected in the plasma, with, as part of the complex movement of ions, a loss of potassium from the cells in exchange for hydrogen ions retained or taken in. This would constitute an "intracellular metabolic acidosis" which, if prolonged, would lead to potassium depletion.

EXCRETORY MECHANISMS

The concentration of the blood and tissue fluid buffers is continuously being maintained by the respiratory and renal mechanisms. The excretion of CO_2 by the lungs not only restores the special haemoglobin mechanisms for re-use, but is capable of adjusting the bicarbonate-carbonic acid system since the rate of excretion of the acid (after conversion to CO_2) depends upon the rate and depth of respiration. The kidneys function by excreting, selectively, acids and hydrogen ions or basic ions in such a way as to vary the urinary *p*H while maintaining the plasma *p*H as nearly constant as possible. Some very weak acids like uric acid are excreted as such, and, being largely undissociated, have little effect on *p*H. Hydrogen ions are excreted to a small extent in that form since normal urine is acid, but mainly in combination with base in the form of NH_4^+ and $H_2PO_4^-$. By this means much hydrogen ion can be removed from the body without a very high acidity being reached in the urine. At the same time, since these ions, excreted by the tubules, must be exchanged for metallic cations, the spent buffer salts are restored. For example, an acid HX is "neutralized" by the buffer systems including the system ($H_2CO_3 + Na^+ + HCO_3^-$), the products being ($Na^+ + X^-$ and H_2CO_3). The carbonic acid may be excreted as CO_2, but the Na^+ and X^- ions are filtered into the glomerular fluid. During the passage of this fluid through the tubules, NH_4^+ and HCO_3^- are produced in the tubular cells, the former from NH_3 (derived mainly from glutamine by enzymic hydrolysis to an extent controlled by the *p*H of the plasma) by combination with H^+ from H_2CO_3 (the end-product of oxidative metabolism). The ammonium ion is then exchanged for the sodium ion in the tubular fluid and, as a net result, $NH_4^+ + X^-$ are excreted in the urine, whilst the reabsorbed Na^+ and the HCO_3^- of the cells are passed back into the plasma, restoring the *status quo*. Similarly, H^+, secreted by the tubular cells, combines with $HPO_4^=$ to form $H_2PO_4^-$ and the Na^+ so released is reabsorbed to take its place.

Tubular lumen

$$Na^+ + Na^+ + HPO_4^= + H^+ \rightarrow Na^+ + H_2PO_4^-$$

$$\downarrow \qquad\qquad \uparrow$$

$$Na^+ \qquad\qquad H^+ + HCO_3^-$$

Tubular cell

The amount of H^+ which can be combined with phosphate in this way is, of course, limited

by the amount of phosphate available for excretion and this is not very great, some 40 mEq. per day; adjustment is by manipulation of the $H_2PO_4^-$: $HPO_4^=$ ratio rather than by change in the total phosphate excretion. The NH_4^+ mechanism is much more flexible because the amount of NH_4^+ obtainable is virtually unlimited; the ordinary excretion of ammonia, some 30–50 mEq./day, may be diminished almost to zero or increased as much as tenfold according to the need for buffer reconstitution.

ACIDOSIS AND ALKALOSIS

Acidosis may be defined as any condition in which the ratio of acid to base in the plasma is increased or in which the concentration of available base, i.e., the buffering power, is reduced. In the first of these situations the *p*H is reduced; the buffering power is not necessarily reduced and may even be above normal. In the second, the processes which produce a decreased buffering power *in vivo* must reduce the *p*H to some extent, but the fall is often so small as to be inappreciable.

Conversely, alkalosis consists of a decrease in the acid : base ratio or an increase in the buffering power.

It is convenient to classify acidosis and alkalosis as respiratory or metabolic. In the former, abnormality of the carbonic acid-bicarbonate system constitutes the primary fault and results from alterations in the CO_2 excretion caused by changes in the respiratory or circulatory mechanism. In the latter, such causes as metabolic over-production of acid, excessive ingestion of base, excessive loss of acid or base by abnormal routes, and failure of the renal excretory processes, alter the buffering power of the body fluids and possibly also the acid : base ratio.

COMPENSATION AND REPAIR

The existence of the homeostatic excretory mechanisms, which tend continuously to adjust the acid : base ratio and the buffering power of the plasma (and hence, by way of internal ion transfer, of the other body fluids), means that any movement towards acidosis or alkalosis automatically provokes a counter-movement. The control of the *p*H seems to be of supreme importance and the initial restorative changes are directed to this end. Frequently the acid : base ratio is returned towards normal (or is prevented from diverging from normal) at the expense of changes in the buffering capacity; a second abnormality is imposed on the first, and the process is one of compensation rather than repair. Thus a respiratory acidosis involving retention of carbonic acid would be repaired by an increased excretion of CO_2; the circumstances in which such an acidosis occurs, however, make such increased excretion difficult or impossible, and in fact the renal mechanisms compensate by raising the acid excretion and permitting an increase in the plasma and tissue buffer concentrations. The apparently anomalous situation thus arises of an acidosis with an increased plasma bicarbonate content; a respiratory acidosis has been compensated by the development of a superimposed metabolic alkalosis. Conversely, a respiratory alkalosis, involving reduction in the plasma H_2CO_3, is compensated by a decrease in the renal excretion of H^+, NH_4^+, and $H_2PO_4^-$ with increased excretion of HCO_3^-—and the corresponding amount of Na^+—with consequent decrease in the plasma [HCO_3^-]. Repair would involve the retention of H_2CO_3.

In a metabolic acidosis, with reduced plasma [HCO_3^-], i.e., reduced buffer capacity, compensation is rapidly initiated by an increased excretion of CO_2 provided the respiratory movements are not affected by some associated disease. True repair, however, involves the much slower adjustment, to whatever extent is possible, of the renal mechanism, with increased output of H^+, NH_4^+, and $H_2PO_4^-$ and the consequent increased reabsorption of Na^+ to allow rebuilding of the plasma concentration of bicarbonate and other buffer salts.

The occurrence of a metabolic alkalosis involves the reverse changes. Again compensation, consisting of the superimposition of increased [H_2CO_3] on the increased [HCO_3^-], is provided by changes in the respiratory movements with retention of CO_2. Again, also, the buffering power of the plasma as well as its *p*H can only be normalized if the kidney can decrease the excretion of H^+, NH_4^+, and $H_2PO_4^-$ allowing the urinary *p*H to rise, and increase the excretion of HCO_3^-, thus decreasing the abnormally high plasma [HCO_3^-]. The excretion of metallic cations like Na^+ will of course alter in harmony with these changes.

In practice compensatory and repair processes proceed simultaneously, their relative rates being determined by the relative efficiencies, in the particular circumstances, of the respiratory and renal mechanisms and by the fact that if both mechanisms are normal the former is the more rapid.

METHODS OF ASSESSING THE STATE OF THE ACID : BASE EQUILIBRIUM

It is clear that abnormalities of the *p*H regulating systems of the body can only be assessed fully by a multiplicity of measurements; there is no single parameter satisfactory in all cases. Moreover, intra- and extra-cellular fluids do not always move in mutual conformity with respect to their acid : base equilibrium, and, since measurements are feasible only in blood or plasma, it is sometimes necessary to infer intracellular abnormalities from indirect evidence.

Since *p*H, an expression of the ratio acid : base, and total buffering power, a function of the base concentration, can vary independently, it is necessary to know both. Quite small changes in *p*H can be very important and therefore only a very accurate *p*H meter can suffice; even with an adequate instrument careful control of temperature is essential, as also is protection against disturbance of the equilibrium by loss of CO_2 to the air. Nevertheless the direct measurement of *p*H is important and is really essential for the proper assessment of respiratory acidosis and alkalosis. Indirect methods of measuring *p*H on the basis, e.g., of determination of arterial plasma $[HCO_3^-]$ and pCO_2, the latter from analysis of the alveolar air with the assumption that its CO_2 content represents the arterial pCO_2, are theoretically possible but unsatisfactory in practice; indeed pCO_2, which is usually abnormal in respiratory disturbances, is best measured by calculation from *p*H and arterial $[HCO_3^-]$. Arterial blood, however, may be replaced by free-flowing capillary blood.

The total buffering power of the plasma could be measured by actual titration to some arbitrary end-point chosen as representing the physiological limit of adjustment. Usually, however, measurement is restricted to the bicarbonate system which, besides being quantitatively the most important in plasma, is the easiest to measure and is in equilibrium with the others. In practice measurement gives the sum of the concentrations of bicarbonate ions, carbonic acid, and dissolved CO_2, but, since the bicarbonate normally constitutes about 95% of this and never, even in extreme acidosis, falls below 90%, little error is involved in accepting "total CO_2 content" as equivalent to $[HCO_3^-]$. The normal range in venous blood is 24–32 mEq. HCO_3^- per litre (50–70 ml. CO_2 per 100 ml.). This measurement is made on venous blood (collected and centrifuged under oil).

For the investigation of acid : base disturbances known to be of metabolic origin with normal respiratory processes, measurement of plasma *p*H is of minor importance and it is determination of buffering power which gives valuable information. It is here that "CO_2-combining power" may profitably be substituted for "total CO_2 content." This is simply the assessment of buffering power by measuring $[HCO_3^-]$ (plus the relatively small $[H_2CO_3 + CO_2]$) in venous blood or plasma which, after withdrawal without precautions against loss of CO_2 to the air, has been re-equilibrated with an atmosphere containing 5.5% CO_2 to represent normal alveolar air. Normally, under these conditions, the re-equilibrated blood on addition of excess acid liberates 54–70 ml. CO_2 per 100 ml. blood; lower figures are found in metabolic acidosis and in compensated respiratory alkalosis, higher figures in metabolic alkalosis and compensated respiratory acidosis.

Investigation of acidosis and alkalosis may include measurement of urinary *p*H and titratable acidity and of urinary ammonia, but these, though occasionally of interest, are generally of minor importance. Cognizance should, however, be taken of the secondary changes in the plasma concentrations of sodium, potassium, and chloride, for these may be important in relation to treatment.

SECONDARY ELECTROLYTE CHANGES

Acidosis and alkalosis invariably consist of changes in the acid : base ratio or in the total buffering power or both. The complex ionic interchanges involved inevitably result in disturbances of those ions which are neither acids nor effective bases, principally Na^+, K^+, and Cl^-. Usually, although the exact mechanism is sometimes obscure, the plasma chloride is lowered, but occasionally, as after ureteral transplantation, acidosis is associated with hyperchloraemia. Sodium depletion may accompany, e.g., the acidosis due to excessive loss of HCO_3^- in severe diarrhoea, for anions cannot be lost without an equivalent loss of cation. Potassium depletion may be a result of intracellular acidosis, for if hydrogen ions produced in the cells are for some reason retained there and react with the buffer systems, an equivalent amount of K^+ leaves the cell and, with normal renal function, is excreted in the urine.

Widespread electrolyte changes and consequently widespread changes in water distribution and excretion must accompany the development and repair of acid : base abnormalities. On the other hand, acidosis and alkalosis may be the result of disturbances among the non-basic and non-acidic ions. One example is the hyper-

chloraemic acidosis mentioned above and another is the acidosis which results from various forms of potassium depletion. The first of these examples is discussed later. In the second, it has been suggested that K^+ and H^+ compete for the same excretory sites in the renal tubular cells and that, therefore, increased excretion of K^+ must lead to some retention of H^+ which, combining with available base, produces acidosis.

CLINICAL CONDITIONS ASSOCIATED WITH DISTURBANCES OF ACID : BASE EQUILIBRIUM

Serious disturbances of the acid : base ratio and of the buffering power are likely to arise in patients suffering from diseases of those organs which are responsible for the regulation of the reaction of the body fluids, namely the lungs and the kidneys. Since the effect of such diseases is usually to diminish the rate of excretion of metabolic end-products, they commonly result in acidosis. Disturbances of regulation can, however, also be caused by loss of alimentary tract secretions, as in vomiting, diarrhoea, or fistula, or by grossly abnormal production of metabolic intermediates, as in uncontrolled diabetes. Acid : base disturbances are also readily produced by the administration of drugs, some acting by virtue of their own chemical nature, e.g., sodium bicarbonate, and others through their action on excretory mechanisms, e.g., the more potent diuretic drugs. The simultaneous occurrence of more than one of these factors is particularly likely to give rise to a clinically recognizable disturbance, as, for example, the patient who vomits repeatedly due to pyloric stenosis but also attempts to relieve his discomfort by frequent ingestion of alkaline mixtures.

(1) Acid : Base Disturbance of Respiratory Type

Carbon Dioxide Retention.—The role of the lungs in acid : base regulation consists in the excretion of carbon dioxide at such a rate as to maintain the arterial CO_2 tension at around 40 mm. Hg, the rate and depth of respiration being regulated towards this end by centres in the brain-stem. Normally the partial pressure of CO_2 in arterial blood equals that in alveolar air, although discrepancies may occur if gases in the lungs are wrongly distributed, as, for example, in open-thorax operations or even in head-down postures.

Upper respiratory tract obstruction as a cause of respiratory acidosis is unusual except in comparatively acute forms, e.g., laryngospasm, inhaled foreign body, and the like, although accumulated bronchial secretions can have a similar effect.

Chronic respiratory acidosis can, however, be readily produced in a variety of ways by pulmonary disease. The patient may have less alveolar tissue than normal, as in emphysema, so that the surface area available for gas exchange is reduced. The residual air after normal expiration may also be considerably increased, so that the inspired air, even if of normal volume, is unable to reduce the alveolar CO_2 tension sufficiently, and in consequence the arterial pCO_2 is high.

Another factor is the elasticity of the lung tissue ; in pulmonary fibrosis, for example, there appears to be not only a diminution in alveolar ventilation, but also poor gas mixing due to lung rigidity preventing elastic recoil.

In pulmonary oedema, the accumulation of fluid reduces the volume of gas available for equilibration with the alveolar capillary blood. Impaired transport of carbon dioxide across the alveolar membrane does not appear to be a significant factor in this condition, since the high solubility of CO_2 permits adequate diffusion, even across an abnormally thick fluid film.

In all these states, therefore, the effect is a reduction in the rate of excretion of carbon dioxide, or, to be more precise, an increase in the gradient of CO_2 tension between blood and inspired air which is required in order to maintain excretion. Carbon dioxide excretion must balance the rate of production in the tissues, otherwise acute asphyxia quickly results, but the arterial CO_2 tension is a reliable index of incipient failure of excretion. There may not always be a demonstrable abnormality in acid : base ratio or in buffering capacity in the resting subject, but mild exertion, or increased tissue metabolism, as in fevers, may precipitate frank respiratory acidosis in patients with chronic pulmonary disease.

It is now becoming clear that in the absence of mechanical obstruction in the air passages, abnormalities of blood flow through the lungs, in relation to the rate of alveolar ventilation, may also produce elevated arterial pCO_2 levels. Over-perfusion of regions of lung, for example, even if the alveolar ventilation rate is normal, results in the return to the systemic circulation of blood with an abnormally high CO_2 tension. Conversely, under-perfusion of normally ventilated alveoli results in an apparent increase in the functional dead space of the lungs, so that the total excretion of carbon dioxide may

be inadequate despite apparently adequate respiratory rate and excursion. These somewhat paradoxical situations involving respiratory acidosis occur mainly in thoracic injuries and in open-thorax operations, and are encountered more often by the anaesthetist than by the general physician.

If the inspired air contains an increased percentage of carbon dioxide, the level at which it is significantly retained is related to the rate of CO_2 production in the body as well as to the composition of the inspired gas. Apart from failure of ventilation in mine-workings, workrooms, and the like, concentrations of CO_2 in the inspired air sufficient to cause respiratory acidosis are seldom encountered except in faulty respirators and anaesthetic apparatus. Some degree of compensation for the high concentration of CO_2 in the alveoli can be attained if the rate and depth of respiration are increased, but obviously no degree of overventilation can achieve excretion against a reversed gradient.

In the presence of normal pulmonary excretory capacity, respiratory acidosis can occur if the sensitivity of the respiratory centres is reduced. Notorious amongst respiratory depressants are such drugs as morphine, the barbiturates, and many anaesthetic agents, but simple hypoxia, especially if prolonged, is also a potent agent in this respect.

Interference with the neuromuscular aspects of respiration can arise in such diseases as bulbar poliomyelitis and progressive muscular atrophy, and also in traumatic lesions affecting the thoracic cage. It may also be produced deliberately in anaesthesia by the use of muscle relaxant drugs such as tubocurarine, and in this latter case it is the responsibility of the anaesthetist to ensure that ventilation is sufficient to prevent the development of respiratory acidosis. Unfortunately there is no simple means of monitoring the arterial CO_2 tension throughout the period of action of the drug, but there is considerable evidence that the state of oxygenation is not a reliable guide to the adequacy or otherwise of controlled respiration. It is probably less harmful to overventilate rather than underventilate the lungs of a patient receiving curare. In the recovery period, when respiration, though spontaneous, tends to be slow and erratic, some degree of respiratory acidosis is almost invariable.

No single laboratory determination is satisfactory as a means of detecting respiratory acidosis and of assessing its severity. Measurement of the CO_2-combining power of venous blood can yield misleading results in this condition, while determinations of the CO_2 content of arterial blood, though informative as regards the buffering capacity, do not reveal the extent of any disturbance of the acid : base ratio. Full information can be obtained from a determination of the *p*H and CO_2 content of a sample of arterial blood collected anaerobically, or alternatively the CO_2 tension of arterial blood may be determined tonometrically and calculations made from this value and the arterial blood *p*H. A third procedure is that of Astrup, who determines the *p*H of whole blood, and of plasma collected anaerobically and then exposed to a gas mixture containing a known percentage of CO_2. From these determinations $[HCO_3^-]$ and $[H_2CO_3]$ can be calculated, or read directly from nomograms. It should be noted that these procedures call for highly accurate determinations of arterial blood *p*H, which in turn require suitable apparatus, including a temperature-controlled bath, preferably at the bedside. Determinations of *p*H made on ordinary laboratory blood samples are futile for this purpose.

Since respiratory acidosis is due to defective excretion of CO_2, compensation and repair cannot be achieved by respiratory means unless the cause is easily remediable. The accumulation of CO_2 causes an increase in the plasma $[H_2CO_3]$, without at first a corresponding increase in $[HCO_3^-]$, so that there is a slight fall in plasma *p*H. This change brings into play the renal mechanisms of compensation, including increased production of NH_4^+ from NH_3 derived from glutamine in the kidney cells. At the same time, hydrogen ion, derived from carbonic acid in the tubule cells, is exchanged for sodium ion in the tubule lumen. The HCO_3^- thus present in the tubule cells enters the plasma (along with the reabsorbed sodium), and it is this entry of base, in the form of bicarbonate ion, into the circulation which restores the acid : base ratio and so returns the plasma *p*H to normal. There is still, however, an abnormally high plasma bicarbonate concentration, and the kidney cannot achieve complete restoration to normal so long as the cause of the respiratory acidosis persists, i.e., its role is compensatory rather than reparative.

Excessive Loss of Carbon Dioxide.—If the rate of CO_2 excretion exceeds the rate of production in the tissues, the partial pressure of CO_2 in the alveolar air falls, with corresponding fall in the $[H_2CO_3]$ in arterial blood. Since the $[HCO_3^-]$ is not at first reduced, the acid : base ratio alters, with a slight increase in blood *p*H, i.e., a

respiratory alkalosis appears. This state of affairs can be rapidly produced by voluntary overbreathing, and since the renal processes of compensation and repair are comparatively slow, a few deep respirations can, in susceptible subjects, produce apnoea, tetany, and even unconsciousness. A similar picture is met clinically in hysteria or in malingerers, but is rarely seen as a manifestation of somatic disease. The rapid respirations of pneumonia do not result in alkalosis, since the respirations are shallow and total alveolar ventilation is, in fact, reduced. Controlled respiration which is too deep or too rapid, as can arise in a poliomyelitis patient treated in a respirator, also results in respiratory alkalosis, since the compensatory mechanism of apnoea is inoperative.

Processes of renal compensation and repair in prolonged respiratory alkalosis are achieved, as one would expect, by the excretion of a neutral or alkaline urine containing an increased concentration of bicarbonate, while the excretion of H^+ as NH_4^+ and $H_2PO_4^-$ is minimized. Changes in the blood in respiratory alkalosis may not be readily detected except by a sensitive *p*H meter, since only a comparatively small reduction in $[H_2CO_3]$ may be present, and determinations of CO_2 content and CO_2-combining power may give results within the normal range, or may be low as the result of compensation.

The principal changes found in the plasma anions in respiratory acidosis and alkalosis are summarized in Fig. 1.

(2) Acid : Base Disturbances of Metabolic Type

Metabolic Acidosis from Excessive Production of Acids.—The processes of normal metabolism result in the production of acidic substances which, if unbuffered in the cells and in the body fluids, would result in intolerably acid conditions. In addition to carbon dioxide (which though quantitatively the most important is, as we have seen, unable to cause acidosis unless excretion is impaired) several other acids are produced as end-products of metabolism, including sulphuric and phosphoric acids. In addition to these end-products, however, there is a considerable number of metabolic intermediates, normally present in small amounts only, and rapidly degraded and re-formed in the course of cell metabolism. This group includes such substances as lactic, pyruvic, citric, and acetoacetic acids, which do not ordinarily enter the extracellular fluid in amounts sufficient to influence acid : base balance directly. One exception is, of course, lactic acid, which

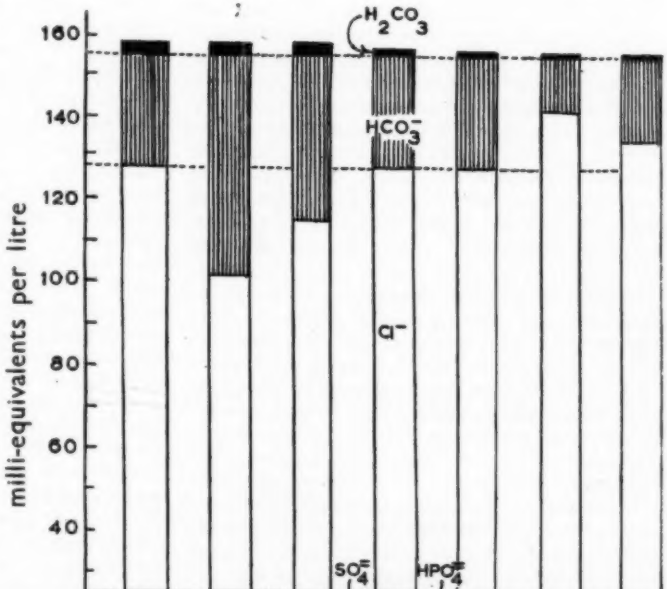

FIG. 1.—Plasma anion changes in respiratory acidosis and alkalosis. In each case, column A shows the anion composition of the plasma characteristic of the primary abnormality ; column B shows the composition when compensation is complete, i.e., the $[HCO_3^-]/[H_2CO_3]$ ratio is restored to normal ; column C shows the composition, with partial compensation, usually found.

accumulates sufficiently during severe muscular exercise to require buffering in the body fluids as well as within the muscle cells.

If, however, there is a metabolic block which results in excessive production of one or more of such acids, or in failure of utilization, the buffering capacity may be exceeded and clinically recognizable metabolic acidosis appears.

The commonest clinical cause of severe metabolic acidosis is uncontrolled diabetes mellitus, in which, owing to impaired carbohydrate utilization in the tissues, there is increased mobilization and partial oxidation of depot and tissue fat. This augments the production in the liver of acetoacetic and β-hydroxybutyric acids, and, owing in part at least to the reduced ability of the peripheral tissues to oxidize such acids to readily disposable CO_2, they accumulate in body fluids. The immediate buffering minimizes the fall in *p*H, and, although there is a resulting increase in $[H_2CO_3]$, this is removed by increased rate and depth of respiration so that the normal acid : base ratio is restored with, however, a lower bicarbonate concentration in the plasma, demonstrable as a reduced CO_2-combining power. Continuing over-production of acids also leads to renal compensation and repair, by increased excretion of hydrogen ion, mainly in combined form as NH_4^+ and $H_2PO_4^-$, and with increased acidity of the urine. There is at the same time an increased urinary loss of sodium, chloride, and water, but these do not directly influence the acid : base disturbance.

In diabetic coma, the plasma electrolyte pattern is as shown in Fig. 2. The arterial blood *p*H is often in the region 7.1–7.3, and the plasma bicarbonate concentration may fall to between 10 and 15 milliequivalents per litre.

A similar, but less severe, metabolic acidosis may be produced by starvation, with increased utilization of body fat. It may also arise from carbohydrate deficiency produced by excessive removal of blood glucose, as in glycogen storage disease.

Metabolic Acidosis from Ingestion of Drugs.—There are a few drugs which, though themselves neutral salts, cause, on oral administration, acidosis of metabolic type. Of these, the best known, and clinically the most useful, is ammonium chloride, but the mechanism whereby acidosis is produced is not at first obvious. Admittedly NH_4^+ is an acid, though a weak one, but since it does not give rise to hydrogen ions at body *p*H, it could not produce acidosis directly. The explanation of its effect lies in the conversion of ammonium ions to urea in the liver, which involves the withdrawal of two HCO_3^- ions from the plasma, and the production of one H_2CO_3 molecule for every molecule of urea formed. The reaction, in greatly simplified form, may be represented as follows:

$$2\,NH_4Cl + 2\,\underset{\text{(from plasma)}}{HCO_3^-} \rightarrow \underset{\text{Urea}}{(NH_2)_2CO} + H_2CO_3 + 2\,Cl^- + 2\,H_2O$$

It is thus seen that ammonium chloride produces a reduction in plasma $[HCO_3^-]$ and an increase in $[H_2CO_3]$. There is also an increase in plasma

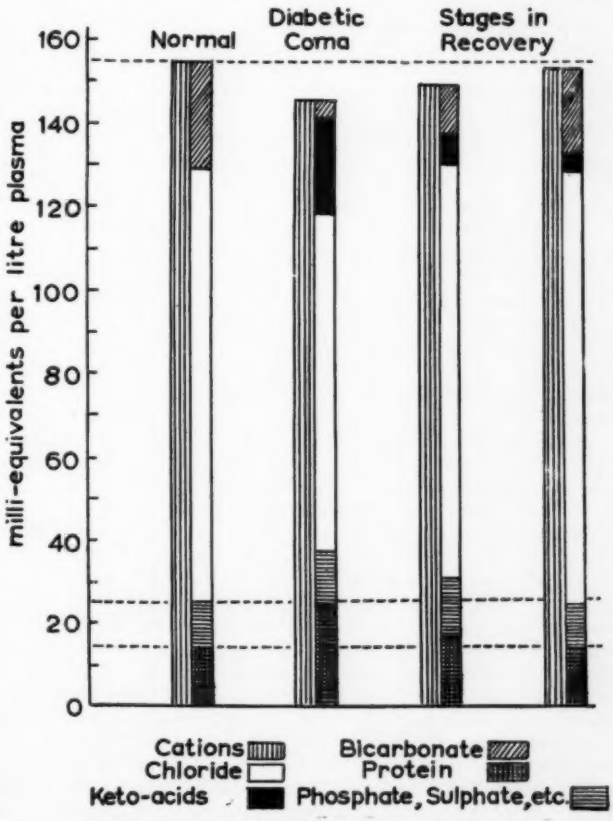

FIG. 2.—Plasma anions in diabetic coma and during recovery.

[Cl^-], but this is a side effect and not a fundamental part of the acidosis. There is no change in osmotic relationships and plasma [Na^+] is not affected.

Metabolic Acidosis Resulting from Failure of Excretion.—Since in normal metabolism acids were continually being produced, failure to excrete a sufficiently acid urine must result in accumulation of H^+ in body fluids. Reconstitution of the buffering capacity cannot be achieved by the lungs, and other routes of excretion (skin, alimentary tract) are insignificant for this purpose.

The acidosis of renal insufficiency has at least two origins. Probably the more important is a diminished production of NH_4^+ in the tubule cells (from glutamine and H_2CO_3), and therefore there is less excretion of hydrogen ion in the form of ammonium ion, as well as less HCO_3^- returned to the plasma to reconstitute the bicarbonate buffer and restore the acid : base ratio. Secondly, there is reduced urinary excretion of phosphate ions, associated therefore with diminished tubular capacity for exchange of H^+ for Na^+, in the conversion of Na_2HPO_4 to NaH_2PO_4 which accompanies tubular acidification of the urine. It is not clear whether the reduced phosphate clearance in renal failure is of glomerular origin, or is secondary to some factor as yet unknown. A further consequence of failure to exchange H^+ for Na^+ is an increased urinary loss of sodium, but this is incidental to, and not part of, the acidosis.

The plasma of a patient in renal failure will therefore show a reduction in [HCO_3^-], normal [H_2CO_3], normal or low [Cl^-], and a characteristic increase in inorganic phosphate concentration.

A special case of partial failure of excretion occurs in patients whose ureters have been transplanted into the lower alimentary canal, and in whom, therefore, reabsorption of urinary constituents can take place. Such patients frequently develop metabolic acidosis which is characteristically associated with diminished plasma [HCO_3^-] but elevated [Cl^-], phosphate and sulphate. Plasma sodium concentrations are normal. This unusual hyperchloraemic pattern does not arise if the urine is not allowed to accumulate in the colon, and isotopic studies have shown that, while both Na^+ and Cl^- are readily absorbed from the colon, the absorption of chloride ion is considerably more rapid. Since an anion cannot be transported without cation, one must either postulate that the absorption of chloride involves an exchange mechanism, with equivalent excretion of another anion, most probably HCO_3^-, or else that a cation other than sodium or potassium, most probably NH_4^+, is also absorbed. The former seems the more likely mechanism, but either process would result in metabolic acidosis by the mechanisms described earlier.

Oral administration of sodium bicarbonate restores normal plasma anion concentrations, and this finding is consistent with either mechanism. In the former case it would act by restoring the plasma [HCO_3^-], and in the latter by acting, via the renal glutamine mechanism, to reduce the urinary excretion of NH_4^+. In both cases, the administered sodium would be excreted along with the surplus chloride, and such patients would not develop oedema from salt retention.

It should be emphasized that the acidosis is not primarily created by the abnormal reabsorption of chloride ion but is produced secondarily by the other ionic transfers which accompany it.

Metabolic Acidosis Resulting from Abnormal Loss of Base.—Direct loss of intestinal secretions, either through prolonged diarrhoea, or from an intestinal fistula, results in loss of HCO_3^- along with equivalent amounts of metallic cations and a certain amount of chloride. Although such losses of Na^+, K^+, and Cl^- cause considerable water and electrolyte depletion, with reduction in the extracellular fluid volume, only the loss of HCO_3^- is responsible for the acid : base disturbance. Once again the plasma pattern shows a reduction in [HCO_3^-], while compensation via the lungs reduces the [H_2CO_3] so that the acid : base ratio is restored at a lower level of bicarbonate buffer concentration. Renal compensation and repair involve, as in other examples of metabolic acidosis, the production of an acid urine having a high concentration of NH_4^+, and an increased excretion of H^+ in the form of $H_2PO_4^-$.

Metabolic acidosis due to increased loss of bicarbonate occurs during the administration of certain diuretic drugs, notably chlorothiazide. The urine, during the diuresis, contains increased amounts of Na^+, K^+, Cl^-, and HCO_3^-, while the urinary *p*H, ordinarily around 5.0–6.0, rises to about 7.5–7.9. Chlorothiazide is a carbonic anhydrase inhibitor *in vitro*, and therefore might be expected to reduce, *in vivo*, the amount of H^+ formed from $CO_2 + H_2O$ in the tubule cells and available for exchange with Na^+ in the tubule lumen. In consequence, the amount of H_2CO_3 entering the blood from the kidney cells would

be greater than normal, while the amount of HCO_3^- entering the plasma would be reduced. It is not yet agreed, however, to what extent the effects of chlorothiazide are attributable solely to its action on carbonic anhydrase activity.

Metabolic Alkalosis Resulting from Excessive Intake of Base. — Antacid drugs used in the treatment of dyspepsia act initially by neutralization of gastric HCl, thereby preventing the subsequent interaction of HCl with $NaHCO_3$ in the intestinal secretions. In consequence, the HCO_3^- of the intestinal secretions is reabsorbed where normally much of it is converted to H_2CO_3 in the gut. If a drug such as sodium bicarbonate is taken in amounts greater than can react with the HCl secreted, there is, in addition, absorption of the HCO_3^- of the drug itself. The plasma $[HCO_3^-]$ is increased, and both CO_2 content and CO_2-combining power are high.

Two restorative processes are possible, respiratory and renal. In the former, the rising blood *p*H reduces the activity of the respiratory centres and the rate and depth of respiration are reduced. Thus the acid : base ratio is restored through an increase in the plasma $[H_2CO_3]$, but at a higher level of buffering capacity than normal. The renal repair processes include diminished NH_4^+ production, diminished exchange of H^+ and Na^+ in the tubules, and increased glomerular filtration of HCO_3^-, the overall result being an increase in urinary *p*H and a reduction of plasma $[HCO_3^-]$.

A metabolic alkalosis may be produced, or an existing metabolic acidosis corrected, by the oral or parenteral administration of certain sodium or potassium salts of weak organic acids. Important examples are lactates and citrates, and for such salts to produce alkalosis it is necessary for the anions to undergo metabolic oxidation in the cells, with the production of carbonic acid. The complete oxidation of one molecule of sodium lactate produces two molecules of carbonic acid and one bicarbonate ion, held in electrical neutrality by the sodium ion of the original lactate. Since the H_2CO_3 can be disposed of by the lungs, the effect is the same as that produced by administration of sodium bicarbonate.

It must again be emphasized that the alkalosis-producing effect of these salts is derived from the strong base HCO_3^- produced by their metabolism, and not from the cation which they provide. The latter may of course be valuable if there is simultaneous sodium depletion, but it does not directly affect the acid : base disturbance.

Metabolic Alkalosis Produced by Loss of Gastric Secretions.—Loss of HCl, as in vomiting or in aspiration of upper alimentary tract secretions, results in metabolic alkalosis, which may be severe if the loss is prolonged. The blood leaving the gastric mucosa during active HCl

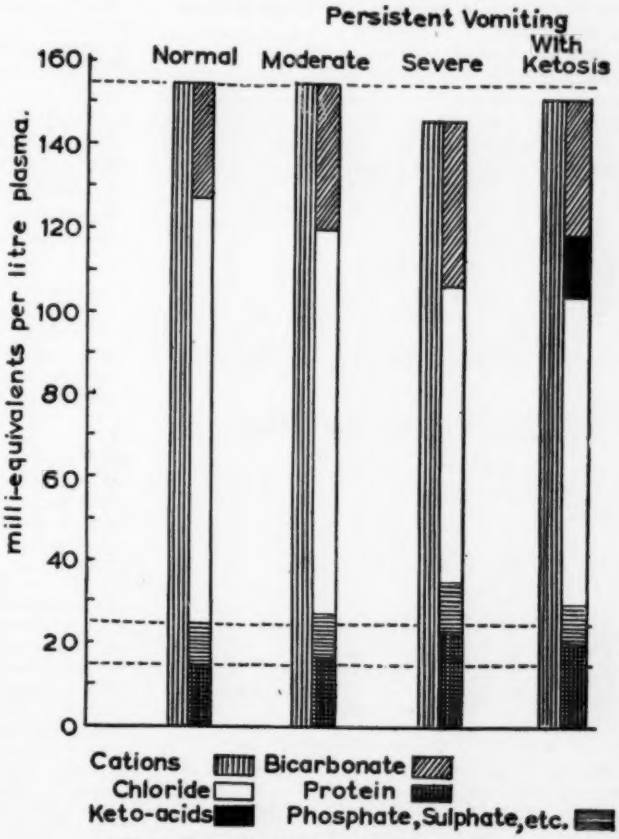

FIG. 3.—Plasma anion changes produced by vomiting.

secretion has a high [HCO_3^-] and low [H_2CO_3], i.e., a temporary metabolic alkalosis occurs which results in transient secretion of alkaline urine following a meal. In the prolonged vomiting of, say, pyloric stenosis, a somewhat similar condition persists, but with progressive diminution in plasma [Cl^-] which is replaced by HCO_3^-. The plasma changes are summarized in Fig. 3. Respiratory compensation through reduced CO_2 excretion is not particularly effective, but the tendency to apnoea may help in the bedside assessment of the severity of the alkalosis. Urinary changes are similar to those which follow administration of bicarbonate, but quantitative measurement of the degree of disturbance can best be made by determination of the plasma [HCO_3^-]. In this context, the determination of the CO_2-combining power of venous blood is all that is necessary, and is preferable to chloride determinations as being a more consistent index of early changes.

Patients suffering from prolonged vomiting usually take an inadequate amount of food, and in consequence tend to increase the breakdown of tissue fat with the production of "starvation ketosis." This condition is, by itself, a cause of metabolic acidosis, and therefore its occurrence in association with severe vomiting may lead to a somewhat confusing plasma anion pattern characterized by normal [HCO_3^-] but low [Cl^-], the deficit being made up by organic anions as shown in Fig. 3. It is thus possible for a patient to be in alkalosis and ketosis simultaneously, and correction of the ketosis, e.g., by parenteral administration of glucose, will commonly reveal the plasma anion pattern characteristic of metabolic alkalosis.

Figs. 1–3 are reproduced from *Clinical Chemistry in Practical Medicine*, by C. P. Stewart and D. M. Dunlop, by kind permission of the publishers, Messrs. E. and S. Livingstone, Edinburgh.

Selected References for Further Reading

(Since the paper describes the application of a particular viewpoint to established facts, a full bibliography is considered unnecessary.)

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ELECTROLYTE EXCRETION IN RESPIRATORY FAILURE

BY

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Patients with chronic emphysema and pulmonary insufficiency commonly arrive at a new steady state in which the carbon dioxide pressure in the blood is increased and the *p*H lowered; this situation can be prolonged for months during which these patients must be in acid-base balance. This equilibrated state of mild respiratory acidosis is, at times, upset by complicating pneumonitis which aggravates the acidosis; this later returns to its previous mild degree, and observations have been made in patients recovering from an acute episode superimposed on chronic lung disease. It was hoped that changes in renal function would be more easily apparent when the degree of acidosis was changing than when it was in equilibrium. In some of these patients the effect of a mild superimposed metabolic acidosis induced by ammonium chloride was observed.

Methods

The 10 patients studied were all men, suffering from chronic bronchitis. Their ages are listed in Table I. Cases 1–7 and 10 were admitted in congestive heart failure, Case 8 in status asthmaticus, and Case 9 with an acute pneumothorax. Cases 1, 4, 5, 6, 8, and 9 had obvious bronchospasm. Case 1 was also hypertensive (blood pressure 220/115 mm. Hg). Most of the patients were being given antibiotics for superimposed pneumonitis and were receiving treatment for congestive heart failure which included low sodium diet and in all but one (Case 8) mercurial diuretics. The general treatment of these patients with antibiotics and with diuretic agents was not altered, and some of the observations may be confused by these facts. Another difficulty has been that of accurately collecting specimens in ill patients, so the results have been "corrected" on the assumption that creatinin excretion did not change in periods of a few hours.

All the patients were judged to be retaining carbon dioxide, with plasma bicarbonate levels either frankly elevated or in the upper normal range (see Table I), and most of them were cyanotic on admission. At the time when observations were made, all patients were improving clinically, but the plasma electrolytes were still abnormal. Cases 3–10 were given ammonium chloride (NH_4Cl) by mouth in a dose of 0.1 g./kg. body weight. An hour later the bladder was emptied, and thereafter, from four to five hours, urine was collected for analysis (Davies and Wrong, 1957). In one patient (Case 7), because of difficulty in emptying the bladder, the period of observation after NH_4Cl had been given had to be extended to 16 hours. In Case 9 the NH_4Cl load was given on two occasions. Plasma and urine samples were analysed in all patients by standard methods in this laboratory (Black and Mills, 1954); the sum of titratable acidity (T.A.), plus ammonium (NH_4^+) less bicarbonate (HCO_3), i.e., hydrion (H^+) excretion was calculated as $(NH_4^+ + T.A.) - HCO_3^-$ (Albright and Reifenstein, 1948). Blood *p*H was measured in Cases 6–10 by an electrometric method at room temperature and corrected to 37°.

Results

Table I shows base line plasma electrolytes. Plasma Na concentrations are generally rather low, K concentrations normal, Cl definitely low, and bicarbonate increased. Possible factors in producing hyponatraemia are the state of the congestive heart failure, the treatment with diuretics and the presence of pneumonitis; the relative importance of these cannot be assessed. The hypochloraemia which is usual in patients

TABLE I

BASE LINE PLASMA ELECTROLYTE CONCENTRATION IN PATIENTS WITH CHRONIC RESPIRATORY INSUFFICIENCY

No.	Name	Age	Na	K	Cl	Total CO_2	Urea	Blood *p*H	Diuretics
1	A.W.	56	139	4·4	88	29	44	—	M
2	D.M.	53	146	4·7	92	33	32	—	M
3	O.M.	55	134	6·5	88	31	24	—	M
4	F.H.	58	131	6·1	76	37·3	44	—	M
5	S.B.	78	131	4·8	88	33	42	7·37	M
6	A.H.	33	136	5·2	84	37·9	23	7·30	M
7	W.B.	75	132	3·5	88·5	32·4	66	7·43	M
8	J.S.	65	143	3·8	101	30·8	—	7·32	—
9	J.B.	59	140	4·7	94·5	36·2	34	7·31	M
9a	J.B.	59	140	4·7	94·5	36·8	34	7·31	M
10	R.H.	54	137	4·6	86	33	47	7·31	M

M = mercurial diuretic.

with respiratory acidosis may have been aggravated by similar factors.

Table II shows the urinary *p*H and the average excretion of NH_4^+, titratable acidity, H^+, and other electrolytes in the control periods, and Table III in the periods after NH_4Cl had been given.

TABLE II

URINARY EXCRETION OF ELECTROLYTES, AMMONIUM, TITRATABLE ACIDITY, AND HYDROGEN ION IN CONTROL PERIOD

No.	Av. Periods	Na (μEq./min.)	K (μEq./min.)	Cl (μEq./min.)	TA (μEq./min.)	NH_4 (μEq./min.)	P (μMol./min.)	H^+ (μEq./min.)	*p*H
1	4	33·3	44·7	29·3	27·5	16·2	—	43·7	5·63
2	2	44·8	34·7	58·8	17·1	24·6	—	41·7	5·60
3	3	44·3	33·0	67·9	20·3	30·5	15·4	50·8	5·84
4	3	16·6	31·4	8·5	29	28·3	25·8	57·3	5·50
5	3	62·6	50·0	51·6	8·8	13·5	18·3	22·3	6·20
6	4	59·1	54·1	53·0	5·3	26·6	28·3	21·3	6·65
7	4	99·3	16·6	87·6	12·8	39·6	8·2	52·4	5·47
8	4	21·8	21·5	38·1	19·2	28·7	12·2	47·9	5·56
9	4	95·4	53·2	91·9	12·8	16·4	25·4	29·2	6·27
9a	1	91·5	40·5	93·9	3·1	13·0	—	16·1	6·5
10	4	44·5	97	64·6	3·6	18·1	—	21·7	6·57

TABLE III

URINARY EXCRETION OF ELECTROLYTES, AMMONIUM, TITRATABLE ACIDITY, AND HYDROGEN ION DURING PERIOD AFTER AMMONIUM CHLORIDE ADMINISTRATION

No.	Na (μEq./min.)	K (μEq./min.)	Cl (μEq./min.)	TA (μEq./min.)	NH_4 (μEq./min.)	P (μMol./min.)	H^+ (μEq./min.)	*p*H
3	142	97·1	244	26·3	46·5	22·6	72·8	5·3
4	39·4	42	50·5	32	49	27·6	81	5·25
5	98	52	89	33·5	24·2	46·6	57·7	5·36
6	168	85	240	27·7	68·5	28·6	96·2	6·06
7	158	17·8	156	13·8	20·5	6·1	34·3	5·21
8	63·7	20·2	80·5	34·6	44·6	27·9	79·2	5·1
9	160	55·2	178	38·5	41·7	29·1	80·2	4·88
9a	178	52	179	39	41·3	—	80·3	5·03
10	48·6	77·5	91·5	46	55	—	101	5·37

There is a striking increase in the excretion of Na, Cl, a less striking increase of P, but not of K. H^+ excretion increased by 130%: titratable activity accounts for 78.8% and NH_4^+ for 51.2% of the increase in H^+. This proportion of NH_4^+ comes within the range found in normal subjects by Wrong and Davies (personal communication).

The relation NH_4^+/pH in all patients but one (Case 6) fell within the normal range (Fig. 1) observed by Davies and Wrong (1957) and Clarke, Evans, MacIntyre, and Milne (1955), a rather surprising result in view of the observed increase in the NH_4 excretion in relation to *p*H in chronic metabolic acidosis (Gamble, Blackfan, and Hamilton, 1924; Sartorius, Roemmelt, and Pitts, 1949; Wood, 1955).

Tables IV and V show the change in the excretion of Na (△Na), K (△K), and K/Cl

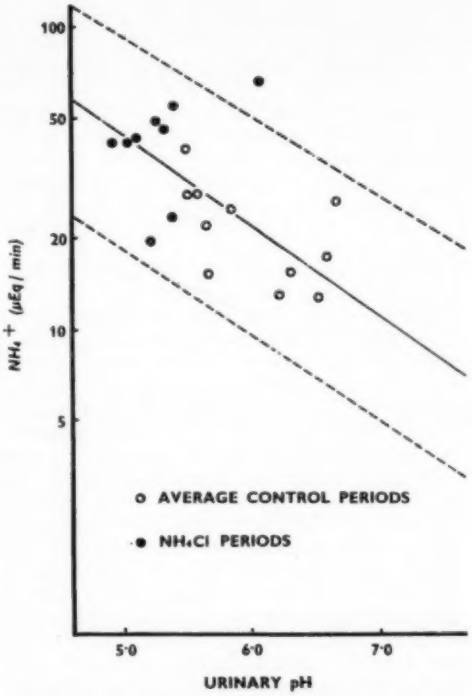

FIG. 1.—Relationship between urinary *p*H and ammonium output in the patients with respiratory acidosis compared with the same relationship in 10 normal controls. The regression line and 95% limits are taken from Davies and Wrong (1957).

relationship (△K/Cl) in 10 normals and in the patients with respiratory failure after NH_4Cl load. There is in relation to normals a significantly decreased Na and K excretion ($t=2.8$, $P\simeq0.02$, and $t=2.7$, $0.05>P>0.02$ respectively) but a decrease that is not significant in the K/Cl relationship ($t=1.7$, $P>0.1$).

TABLE IV

URINARY SODIUM, POTASSIUM, AND POTASSIUM CHLORIDE RATIO CHANGES AFTER AMMONIUM CHLORIDE ADMINISTRATION IN NORMALS (UNPUBLISHED DATA BY WRONG AND DAVIES)

Case No.	△Na	△K	△K Cl
1	142	114	0·38
2	392	44	0·14
3	198	62	0·21
4	139	65	0·23
5	119	−9	0·00
6	88	41	0·38
7	90	62	0·24
8	77	28	0·24
9	76	3	0 00
10	107	54	0 35

TABLE V

URINARY SODIUM, POTASSIUM, AND POTASSIUM/CHLORIDE RATIO CHANGES AFTER AMMONIUM CHLORIDE ADMINISTRATION IN PATIENTS WITH CHRONIC RESPIRATORY INSUFFICIENCY

Case No.	Na	K	K/Cl
3	98	64	0·38
4	23	11	0·26
5	35	2	0·07
6	109	31	0·17
7	17	1	0·01
8	22	−1	−0·02
9	74	3	0·04
9a	86	11	0·14
10	4	−19	−0·70

Discussion

As would be expected, the results show an increase in H^+, Na, Cl, and P excretion after NH_4Cl loading. A logarithmic relationship between the rate of ammonium excretion and the urinary *p*H has already been shown (Clarke *et al.*, 1955). The present results show that all but one of the patients with chronic respiratory insufficiency have an NH_4^+/*p*H relationship in the normal range (Clarke *et al.*, 1955; Davies and Wrong, 1957) even after NH_4Cl loading. The ammonium excretion in chronic metabolic acidosis is high in relation to the urinary *p*H (Gamble *et al.*, 1924; Sartorius *et al.*, 1949; Wood, 1955) and this has been attributed to a high glutaminase activity in the renal cortex induced by chronic acidosis (Davies and Yudkin, 1952). It seemed likely that in chronic respiratory acidosis the same renal response might occur, but in fact a normal NH_4^+/*p*H relationship has been found in almost all the patients. In the light of the recent work published by Carter, Seldin, and Teng (1958), these results can be interpreted as a consequence of the lack of increase in renal glutaminase activity in respiratory acidosis.

It has been shown in normal subjects that there is a rapid fall of urinary *p*H after NH_4Cl (Clarke *et al.*, 1955; Davies and Wrong, 1957); values higher than 5.3 after NH_4Cl are commonly interpreted as caused by renal tubular acidosis or K deficiency (Davies and Wrong, 1957). The average urinary *p*H in the patients after NH_4Cl had been given was 5.28, which is near the upper limit of the normal range given by Davies and Wrong (1957) and Clarke *et al.* (1955).

In the patients with respiratory failure the excretion rate of urinary K and Na after NH_4Cl increased less than it did in normal subjects given NH_4Cl. In respect of Na this can be attributed partly to low salt intake and partly to congestive heart failure. The K intake, however, was normal, and congestive heart failure is not usually associated with low excretion of K.

Clarke *et al.* (1955) have shown that, when normal persons are made K-deficient, their K output falls, Na increases, and K/Cl after NH_4Cl is low; and the low K output in these patients may well be due to K depletion. Potassium depletion has not been generally recognized as a complication of congestive heart failure secondary to respiratory insufficiency, but Black and Davies (unpublished observations) found K retention in two patients recovering from acute respiratory failure. More recently Levitin, Jockers, and Epstein (1958) and Carter *et al.* (1958) have shown a decrease in muscle K in rats exposed to high CO_2 tension for a long time. Although the present results may suggest K depletion they do not indicate whether this is due to the acidosis, to the state of heart failure, or to the effect of treatment with a low salt diet and diuretics. The determination of the changes in K and Na excretion after NH_4Cl might be of interest in other types of congestive heart failure in which K depletion may coexist with a normal level of plasma K, and urinary output of K within the wide normal range.

Summary

The urinary excretion of ammonium, titratable acidity, and electrolyte has been estimated in 10 patients with respiratory failure. In eight an NH_4Cl load was given. In all cases but one, ammonium excretion bore the normal relationship to urinary *p*H. After ammonium chloride administration, the increase of sodium and potassium excretion was significantly smaller in these patients than in normal subjects; this finding is discussed.

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GASTRIC PROTEOLYSIS IN DISEASE

1. THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE FROM PATIENTS WITH PERNICIOUS ANAEMIA

BY

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Fenwick (1870) was the first to discover that patients with pernicious anaemia have a deficiency of gastric pepsin, and this observation has been amply confirmed (Stockton, 1904; Levine and Ladd, 1921; Teschendorf, 1927; Faber, 1927; Faber and Holst, 1928; Johansen, 1929; Polland and Bloomfield, 1930; Castle, Heath, and Strauss, 1931; Davies, 1931; Wilkinson, 1932; Helmer, Fouts, and Zerfas, 1932; Hartfall, 1933; Maltby, 1934; Griffiths, 1934; Mullins and Flood, 1935; Ihre, 1938; Witebsky, Klendshoj, and Vaughan, 1942; Janowitz and Hollander, 1951; Aitken, Spray, and Walters, 1954). Pepsin secretion is usually either absent or slight, whether the gastric juice is obtained in the resting state or after stimulation with histamine or insulin. Low values are still obtained when the stomach is washed with dilute HCl during the collection of juice in order to prevent the inactivation of pepsin at the neutral *p*H of gastric secretion in pernicious anaemia (Ihre, 1938). Occasionally patients have been observed who, despite achlorhydria, secrete appreciable though still subnormal amounts of pepsin (Faber, 1927; Davies, 1931; Hartfall, 1933; Griffiths, 1934; Jones and Wilkinson, 1938; Aitken, Spray, and Walters, 1954; Taylor, 1956).

The proteolytic activity of the gastric juice in pernicious anaemia has also been investigated at neutral *p*H. In contrast to the mild proteolysis observed with normal gastric juice, no activity or decreased activity has been reported by Griffiths (1934), Helmer, Fouts, and Zerfas (1932), Lasch (1937), and Taylor, Castle, Heinle, and Adams (1938). Slight activity, which does not significantly differ from that of normal subjects, has been observed by Maltby (1934), Emerson and Helmer (1936), Jones, Grieve, and Wilkinson (1938), and Gessler, Dexter, Adams, and Taylor (1940).

In all this work there has been an underlying assumption that the differences which exist between normal gastric juice and that from patients with pernicious anaemia result from a simple quantitative deficiency of normal proteolytic activity. Usually determinations of proteolytic activity have only been carried out at *p*H 2.0 or *p*H 7.4. Only one *p*H activity curve for proteolysis by gastric juice from a patient with pernicious anaemia has been reported (Jones and Wilkinson, 1938). This showed a single maximum at *p*H 2.2, but no determinations were made between *p*H 3 and 4 where a second peak occurs in normal subjects (Taylor, 1959a). The possibility that the gastric juice in pernicious anaemia may contain different enzymes from normal gastric juice or differing proportions of normal enzymes does not seem to have been considered. An investigation of this possibility is now described.

Experimental

Determinations of proteolytic activity were carried out by formol titration using the technique already described (Taylor, 1957, 1959a). Samples of gastric juice from patients with pernicious anaemia were usually secreted at *p*H 6 to 8, brought quickly to a *p*H of about 4 with 0.1 M HCl, and stored at 0 to 4° C. until used.

Pernicious anaemia was diagnosed clinically by the presence of a macrocytic hyperchromic anaemia, a megaloblastic bone marrow, and a histamine-fast achlorhydria in the absence of clinical and laboratory evidence of steatorrhoea, and was confirmed by a positive response to treatment with vitamin B_{12}. In several patients the presence of subacute combined degeneration of the cord, the findings of a gastric biopsy, and the inability to absorb more than 40% of an oral dose of (^{60}Co)-vitamin B_{12} have increased the certainty of the diagnosis.

Results

Action on Protein Substrates below *p*H 5.—Twenty-six samples of gastric juice from 23

TABLE I

PROTEINASE ACTIVITY BELOW *p*H 5 OF GASTRIC JUICE FROM PATIENTS WITH PERNICIOUS ANAEMIA*

Patient	Substrate	*p*H 1·5 to 2·4		*p*H 2·5 to 3·2		*p*H 3·3 to 4·0		*p*H 4·1 to 5·0	
		Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)
1. Histamine juice ..	Plasma protein	2·4	0·09	—	—	3·7	0·07	Inactive	
2. ,, ,, ..	,, ,,	Inactive		Inactive		Inactive		,,	
3. ,, ,, ..	,, ,,	,,		,,		,,		,,	
,, ,, ..	,,	,,		,,		,,		,,	
4. ,, ,, ..	Egg albumen	,,		,,		,,		,,	
5. ,, ,, ..	Plasma protein	2·0	0·35	3·0	0·32	Declining activity		Declining activity	
	Egg albumen	1·6	0·20	Declining activity		Inactive		Inactive	
	Plasma protein	1·8	0·10	2·6	0·08	,,		,,	
6. ,, ,, ..	,, ,,	1·9	0·06	3·2	0·07	Declining activity		,,	
7. ,, ,, ..	,, ,,	2·0	0·08	3·2	0·11	,,	,,	,,	
8. ,, ,, ..	,, ,,	Declining activity		2·7	0·29	,,	,,	,,	
9. Insulin ,, ..	,, ,,	,,	,,	3·2	0·08	,,	,,	,,	
10. Histamine ,, ..	,, ,,	,,	,,	2·8	0·11	,,	,,	,,	
11. ,, ,, ..	,, ,,	2·0	0·04	Declining activity		Inactive		,,	
	Egg albumen	Inactive		,,	,,	3·7	0·13	Declining activity	
12. ,, ,, ..	Plasma protein	,,		,,	,,	3·6	0·09	,,	,,
,, ,, ..	,, ,,	,,		,,	,,	3·5	0·06	,,	,,
13. ,, ,, ..	,, ,,	,, †		,,	,,	3·7	0·10	,,	,,
,, ,, ..	,, ,,	,,		,,	,,	3·5	0·07	Inactive	
14. ,, ,, ..	,, ,,	Declining activity		2·7	0·23	Declining activity		,,	
15. ,, ,, ..	Serum albumin	2·3	0·18	Declining activity		Inactive		,,	
16. ,, ,, ..	,, ,,	Inactive		,,	,,	Declining activity		4·4	0·09
17. ,, ,, ..	Plasma protein	,,		,,	,,	,,	,,	4·2	0·07
18. ,, ,, ..	,, ,,	,, †		,,	,,	,,	,,	4·3	0·09
19. ,, ,, ..	,, ,,	Declining activity†		,,	,,	,,	,,	4·6	0·10
20. ,, ,, ..	,, ,,	Inactive		,,	,,	,,	,,	4·1	0·05
21. ,, ,, ..	,, ,,	2·4	0·07	—	—	—	—	4·2	0·05
22. ,, ,, ..	,, ,,	Declining activity		2·6	0·12	3·8	0·10	Declining activity	
23. ,, ,, ..	Serum albumin								

* In the tables and figures the amounts of amino-acid nitrogen released are those in the whole digest, which usually consisted of 3 ml. of buffer, 0·5 to 2 ml. of gastric juice, and 1 ml. of plasma or 5% (w/v) albumin. 37° C., 3 hr.
† No determinations below *p*H 2·0.

patients were incubated with plasma protein, human serum albumin, or egg albumen at *p*H 1.5 to 5.0 for 3 hr. at 37° C. (Table I, Figs. 1 and 2). Only one patient (No. 1) exhibited the two normal maxima, falling within the *p*H ranges 1.7 to 2.4 and 3.3 to 4.0, which have been established for normal gastric juice under similar experimental conditions (Taylor, 1956, 1959a). The remaining

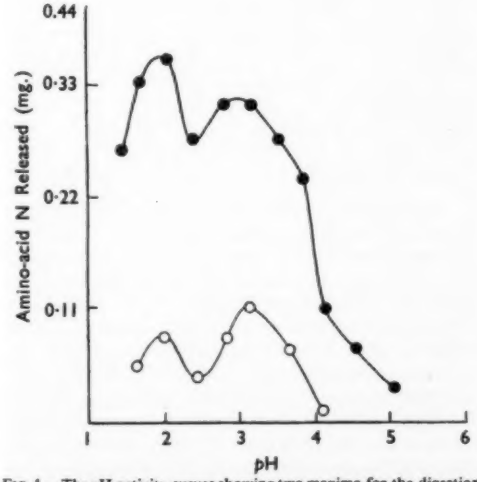

FIG. 1.—The *p*H activity curves showing two maxima for the digestion of plasma protein by the gastric juice of two patients with pernicious anaemia (incubated at 37° C. for 3 hr.).

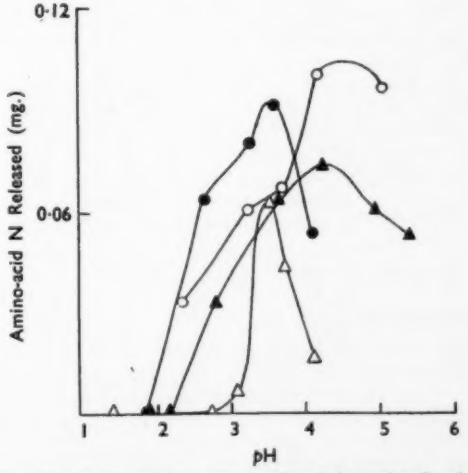

FIG. 2.—The *p*H activity curves showing single maxima for the digestion of plasma protein by the gastric juice of four patients with pernicious anaemia (incubated at 37° C. for 3 hr.).

patients fall into four main groups. Group I consists of three patients (Nos. 2, 3, and 4) in whom there was no demonstrable proteolytic activity of the gastric juice. Group II consists of four patients (Nos. 5 to 8) who exhibited two *p*H maxima but in whom the upper maximum occurred at *p*H 2.6 to 3.2. Group III comprises eight patients (Nos. 9 to 16) whose gastric proteolytic *p*H activity curves exhibited only a single maximum between *p*H 2.0 and 3.7. Group IV consists of five patients (Nos. 17 to 21) who exhibited only a single maximum at *p*H 4.1 to 4.6. In one patient from Group III and two from Group IV there was insufficient gastric juice to extend the *p*H activity curve below 2.0, so that the possibility of a second peak below this *p*H cannot be excluded. Apart from patients 5, 9, and 15, the amount of activity at the *p*H maxima was slight in comparison with that of normal subjects.

Action on Proteins above *p*H 5. — The histamine-stimulated gastric juices of 15 patients were incubated with plasma protein or human serum albumin at *p*H 5 to 8 (Table II). In all but one patient proteolytic activity was detected. *p*H maxima were exhibited at *p*H 6.8 to 7.1 in three patients, at *p*H 7.6 in eight patients, and at *p*H 7.9 in one. In this last patient the activity was high and the gastric juice also exhibited carboxypolypeptidase activity upon chloracetyl-L-tyrosine, indicating contamination with pancreatic juice.

Maxima sometimes occur with normal subjects at *p*H 6.8 to 7.2 (seven out of 14) and at *p*H 7.6 (three out of 14) (Taylor, 1959d) and it has been shown that the former is caused by an enzyme in gastric juice, but that the latter probably results from contamination with a salivary enzyme. The observations in pernicious anaemia differ therefore from those of normal subjects only in the proportions of patients exhibiting these two maxima. They do not reveal the existence of abnormal *p*H activity curves.

TABLE II

PROTEINASE ACTIVITY AT *p*H 5 TO 8 OF HISTAMINE GASTRIC JUICE FROM PATIENTS WITH PERNICIOUS ANAEMIA INCUBATED AT 37° C. FOR 3 HR.

Substrate / Patient	Maximal *p*H	Amino-acid N Liberated (mg.)
Plasma protein		
2.	6·8	0·12
	7·6	0·09
3.	7·6	0·13
4.	Inactive	
20.	7·6	0·15
24.	7·6	0·06
25.	6·9	0·20
26. (Bile-stained) ..	7·6	0·20
27. ,, ..	7·6	0·29
28. ,, ..	7·6	0·13
29. ,, ..	7·6	0·16
30. ,, ..	Active, no maximum	
Serum albumin		
24.	,, ,, ,,	
31.	7·1 ,, ,, ,,	0·07
32.	Active, no maximum	
33.	,, ,, ,,	
34. (Bile-stained) ..	7·9 ,, ,, ,,	0·67
35. ,, ..		

TABLE III

EFFECT UPON PROTEOLYTIC ACTIVITY OF NORMAL GASTRIC JUICE AT *p*H 2·5 OF ADDING TO DIGESTION MIXTURE GASTRIC JUICE FROM PATIENTS WITH PERNICIOUS ANAEMIA

	Formol Titration			
Patient No. :	12	18	21	4
4 ml. buffer + 1 ml. plasma + 1 ml. normal gastric juice + 2 ml. water. Incubated 37° C., 3 hr. / 1 ml. buffer + 1 ml. P.A. gastric juice. Incubated 37° C., 3 hr. (Mixed)	11·93	12·09	12·35	12·22
5 ml. buffer + 1 ml. plasma + 1 ml. normal gastric juice + 1 ml. P.A. juice. Incubated 37° C., 3 hr. Then 2 ml. H_2O added ..	12·12	12·25	12·31	12·22

In a preliminary experiment 1 ml. of normal gastric juice incubated with 1 ml. plasma had produced an increased formol titration of 0·53 ml. N/10 NaOH. The values in the table are the means of duplicate estimations. Formol titrations expressed in ml. N/10 NaOH.

Presence or Absence of an Inhibitor of Gastric Proteolysis.—A possible explanation of the occurrence of *p*H activity curves with single maxima in the region of *p*H 2.7 to 4.6 might be the presence in gastric juice in pernicious anaemia of a substance inhibiting peptic action at *p*H 1.5 to 2.5. This hypothesis has been tested at *p*H 2.5 by incubating normal human gastric juice with plasma protein alone and with gastric juice from each of four patients with pernicious anaemia. In each instance there was no decrease of formol titration in the tube containing pernicious anaemia juice and thus no evidence of inhibition of normal gastric proteolysis was obtained (Table III).

Proteolytic Activity of Normal Duodenal and Ileal Mucosal Extracts.—Biopsy and necropsy studies show that the gastric mucosa in pernicious anaemia occasionally contains patches of cells showing intestinal metaplasia. If these cells were to liberate a proteinase into the gastric juice, an unusual *p*H activity curve might appear. An attempt to evaluate this source of error was made by determining the proteinase activity of extracts of duodenal and ileal mucosa which had been removed at operation from patients with carcinoma of the stomach and ascending colon. The duodenal extracts exhibited two maxima (Taylor, 1959e) similar to those exhibited by

pyloric extracts (Taylor, 1959c) but quite different from the abnormal single maxima that occur in pernicious anaemia. Two ileal extracts, however, upon incubation with plasma protein, exhibited mild proteinase activity with a single maximum at *p*H 3.5 to 3.7. The possibility cannot therefore be excluded that abnormal curves with single maxima in this *p*H range result from secretion into the gastric juice of a proteinase derived from cells showing intestinal metaplasia, although proof of such a secretion, or of the identity of the ileal and the metaplastic cells, is lacking.

Discussion

The complete absence of proteolytic activity in the patients of Group I is not surprising in view of the existing evidence that peptic activity at *p*H 2 is frequently absent from the gastric juice of patients with pernicious anaemia. The unexpected feature is that the proportion of inactive juices was so low. However, had peptic activity been determined solely at *p*H 2, only 13 of 23 patients (Table I) would have been recorded as secreting pepsin in even minimal amounts. Estimations at *p*H 2.0 give, therefore, a false picture of the proteolytic power of the gastric juice in pernicious anaemia, for when activity was sought at *p*H 2.6 to 5.0 it was detected in as many as 20 out of 23 patients.

Those patients whose gastric juices were active below *p*H 5 exhibited a wide variety of *p*H activity curves. In order to interpret the abnormal curves, it is necessary to recollect certain features of the proteolytic activity of normal gastric juice. It has been established previously (Taylor, 1956, 1959a) that, under the same experimental conditions as those described in this paper, normal human gastric juice will digest plasma protein and human serum albumin below *p*H 5 with two maxima in the *p*H ranges 1.7 to 2.4 and 3.3 to 4.0. Egg albumen is digested with only one maximum at *p*H 1.5 to 1.8. It has also been shown (Taylor, 1959b) that the two maxima result from the presence in the pepsin molecule of two different types of active centre, and that at least two different pepsin molecules, each exerting two proteolytic maxima below *p*H 5, occur in normal gastric juice (Taylor, 1959c). One of these molecules preponderates in pyloric mucosa and the other in the mucosa of the body and fundus of the stomach. The "pyloric" pepsin exhibits maximal activity at *p*H values of 1.5 to 2.0 and 2.6 to 3.2, which are lower than those for normal gastric juice or for fundic mucosal extracts.

The patients of Group II thus exhibit *p*H activity curves which closely resemble those obtained with extracts of human pyloric mucosa. One hypothesis that would account for such curves is that the peptic activity in these four patients is derived from pyloric mucosal cells. This hypothesis is supported by the knowledge that in man (Fox and Castle, 1942) the fundus and body of the stomach contain intrinsic factor whereas the pyloric mucosa does not, and that in pernicious anaemia the fundus and body mucosa degenerates but the pyloric mucosa is unaffected (Magnus and Ungley, 1938). Were this the case in these patients, the gastric juice secreted after histamine stimulation would be principally derived from the pyloric mucosa and would be expected to exert proteolytic action upon plasma protein with the *p*H maxima that have in fact been found.

Three hypotheses may be put forward to explain the occurrence of abnormal *p*H activity curves with single maxima. The first of these, the presence of a substance inhibiting one of the two normal *p*H maxima, must, from the experimental evidence, be abandoned. The second, that cells showing intestinal metaplasia secrete an abnormal enzyme, could only account for the curves with maxima at *p*H 3.5 to 3.7, observed in three patients. The hypothesis would not account for the existence of *p*H activity curves with single maxima below 3.2 or above 4.0. The third hypothesis is that the gastric mucous membrane in pernicious anaemia may secrete abnormal gastric proteinases, i.e., enzymes which have a different chemical structure from the two normal pepsins. The curves of Group III would be explained if such abnormal enzymes consisted of fragments of normal pepsins in which only one active centre was present or if they were comprised of more or less normal pepsins in which one active centre was absent. The curves of Group IV would, however, only be explained by the production of a pathological enzyme with a different active centre from those normally present. This last explanation is the only one that would satisfy all the experimental observations of Groups III and IV.

The concept of the production by diseased tissues of abnormal and pathological enzymes will need to be confirmed by other evidence, such as the isolation and characterization of the enzymes themselves, before it can be regarded as firmly established. It should be noted, however, that the pathological features of pernicious anaemia are compatible with the possible elaboration and secretion of abnormal enzymes. Thus the gastric

mucous membrane is degenerated and produces enzymes in an environment that is deficient in vitamin B_{12}, in folinic acid, and perhaps also in oxygen. The disordered protein synthesis which the elaboration of an abnormal enzyme implies might well arise under such conditions. Lastly, it may be pointed out that, although the existence of abnormal enzymes in disease is not well recognized, the elaboration of abnormal proteins is widely accepted, for example, the Bence Jones protein of multiple myelomatosis, the production of cryoglobulins in liver disease, or the presence in many diseases of abnormal haemoglobins in erythrocytes.

Summary

The proteolytic *p*H activity curves for gastric juice from patients with pernicious anaemia have been determined below *p*H 5 in 23 patients and between *p*H 5 and 8 in 15.

Below *p*H 5 only one patient exhibited a normal curve with two proteolytic maxima in the *p*H ranges 1.5 to 2.4 and 3.3 to 4.0.

Of the remaining patients, three secreted inactive juices, four secreted juices with activity resembling that of normal pyloric mucosal extracts, and 13 secreted juices exhibiting only single maxima. In five of these 13 patients the single maximum occurred above *p*H 4 and thus at a point where normal gastric proteolytic activity declines rapidly.

The abnormal *p*H activity curves are tentatively explained as follows:

(*a*) In those patients who show no activity, the fundic and pyloric glands are completely atrophic.

(*b*) In those who show the pyloric type of activity, the pyloric mucous membrane is preserved.

(*c*) In those who show abnormal maxima, abnormal enzymes are elaborated and secreted.

Determination of proteolytic action only at *p*H 2 gives an underestimate of the proteolytic power of the gastric juice in patients with pernicious anaemia.

The gastric juice of only one of 15 patients failed to show proteolytic activity at *p*H 5 to 8. Maxima occurred in three patients at *p*H 6.8 to 7.1 and in eight at *p*H 7.6. In normal subjects maxima occur in the same *p*H ranges, but that at *p*H 6.8 to 7.1 is the more common.

Some implications of the concept that abnormal enzymes may occur in human disease are considered.

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Addendum

Since this paper was written, Martin (1958) has drawn attention to the change of substrate pattern for serum pseudocholinesterase found in liver disease by Kekwick (1955) and has pointed out that one explanation of these results might be the release from the damaged liver cell of a "'broken-backed' enzyme."

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AN EVALUATION OF THE EUGLOBULIN METHOD FOR THE DETERMINATION OF FIBRINOLYSIS

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Methods for determining fibrinolysis are not satisfactory. The reproducibility and accuracy of many known methods are doubtful, and it is difficult to say how far they reflect the processes and the fibrinolytic potential in the living organism, so in this paper will be presented the results of many years' experience with the euglobulin method.

Methods and Materials

Reagents.—The reagents are 0.1 M ammonium oxalate in distilled water, and a borate solution made up of 9 g. sodium chloride and 1 g. sodium borate in 1,000 ml. distilled water.

Streptokinase was purchased from Warszawska Wytwórnia Surowic i Szczepionek.

Fibrinogen was prepared by the method of Kekwick, Mackay, Nance, and Record (1955).

Thrombin was prepared after separating prothrombin by the method of Lewis and Ferguson (1953) slightly modified. Prothrombin was converted to thrombin using the calcium-thromboplastin mixture, and thrombin was precipitated by acetone and extracted from the acetone-dried powder with distilled water.

The euglobulin precipitation is based on an early observation of Milstone (1941) and Macfarlane and Biggs (1948) that plasminogen is a chief component of the euglobulin fraction. The method, as used here, has been worked out by Kowarzyk and Buluk (1950).

Method.—Blood was collected into 0.1 M ammonium oxalate in the proportion of 1 part of anticoagulant to 9 parts of blood, and plasma was obtained by centrifuging for 10 minutes at 1,500 r.p.m. To 0.5 ml. of plasma 8 ml. distilled water and 0.15 ml. 1% acetic acid were added. The *p*H should be approximately 5.2. After 30 minutes' incubation in a refrigerator at +4° C. the tubes were centrifuged for five minutes at 1,500 r.p.m. The supernatant was poured off and the tubes were drained by inverting on filter paper for about one minute. The precipitate was dissolved with the aid of a fine glass rod in 5 ml. borate solution, and the tubes were placed in a water-bath at 37° C. After a few minutes 0.5 ml. 0.025 M calcium chloride was added. The clot which formed within a few minutes was observed every 10 minutes and the lysis time recorded.

As shown by Copley, Niewiarowski, and Marechal, in co-operation with this laboratory, it is possible to utilize this method for volumes of plasma as small as 50 μl.

Other Determinations.—Fibrinolysis in whole plasma and recalcification time were determined by the method of Biggs and Macfarlane (1957); total protein in plasma and in euglobulin fractions was determined by the method of Weichselbaum (1946); trichloracetic acid-soluble tyrosine was determined by the method of Folin and Ciocalteu (1927), fibrinogen and the prothrombin index were determined by the methods of Quick (1935, 1957); plasminogen was determined by the method of Kowalski, Latallo, and Niewiarowski (1956); antiplasmin by the method of Niewiarowska (1957); two-stage prothrombin by the method of Leonow (1950); Factor V by the method of Wolf (1953); and Factor VII by the method of Koller, Loeliger, and Duckert (1951); antihaemophilic globulin and Christmas factor were determined by the method of Pitney (1956).

Results

To evaluate the usefulness of the euglobulin method for the clinician, results obtained by examining 292 normal subjects were submitted to a statistical analysis carried out in the course of seven years by different workers. It can be seen from Table I that the mean lysis time for several sets of results showed only negligible variations from 210 to 221 minutes. The frequency shows a Gaussian distribution (Fig. 1).

TABLE I

FIBRINOLYSIS IN EUGLOBULIN OF NORMAL SUBJECTS

Authors	No. of Cases	Mean (min.)	Standard Deviation	Standard Error	Maximal Value	Minimal Value
Kowarzyk and Buluk (1950)		210				
Kopeć *et al.* (1952)	90	216	±67	±7	340	20
Kopeć and Niewiarowski (1956) ..	156	219	±61	±5	365	45
Kowalski, Kopeć, and Niewiarowski (Present series)..	46	221	±54	±7·97	340	46

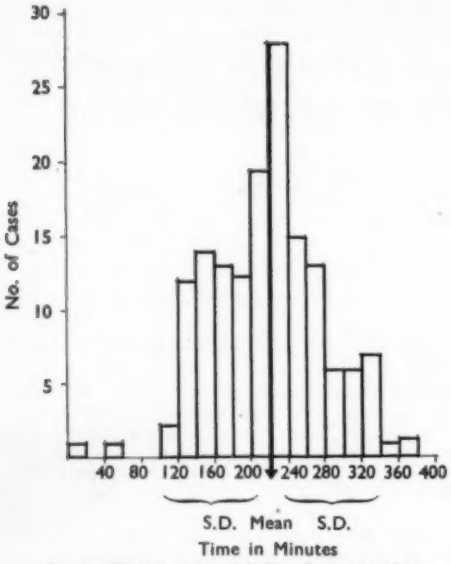

FIG. 1.—Fibrinolysis in euglobulins of normal subjects.

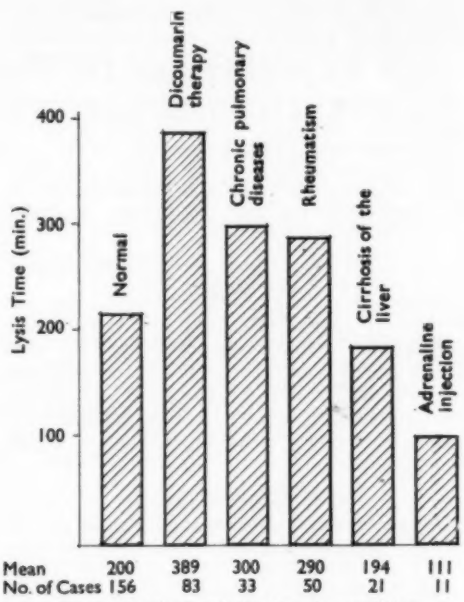

FIG. 2.—Euglobulin lysis time in various conditions.

To determine the usefulness of this method in demonstrating disturbances of fibrinolysis in various conditions, results obtained in the investigation of selected groups of subjects were analysed. It can be seen from Fig. 2 that in normal subjects after adrenaline injection the euglobulin fibrinolysis time is shortened. In rheumatoid arthritis and in chronic lung disease (Kopeć, Niewiarowski, and Kowalski, 1952), fibrinolysis time is lengthened. In patients on dicoumarol therapy fibrinolysis time is lengthened; this has been shown in a separate paper by Niewiarowska and Wegrzynowicz (1958). Patients with cirrhosis of the liver show only insignificant shortening of euglobulin fibrinolysis time.

To obtain more information about the theoretical basis of the euglobulin method certain biochemical studies were performed. Table II shows the most important components and activities of the coagulation and fibrinolysis system in euglobulin solution compared with plasma.

It can be seen that the euglobulins, which amount to 10% of the total plasma proteins, contain all the plasminogen, factor V, and anti-haemophilic activity, but that the amounts of

TABLE II

SOME FEATURES OF COAGULATION AND FIBRINOLYTIC SYSTEM OF EUGLOBULINS COMPARED WITH PLASMA

Component or Activity Tested	% of Plasma Activity
Total protein	10
Fibrinogen	25
Plasminogen	100
Antiplasmin	Traces
Recalcification time	Normal
Prothrombin index	50
Two-stage prothrombin	56
Factor V	100
,, VII	20
Antihaemophilic globulin	100
Christmas factor	Traces

FIG. 3.—Proteolysis and fibrinolysis in euglobulins after the addition of $CaCl_2$ or streptokinase.

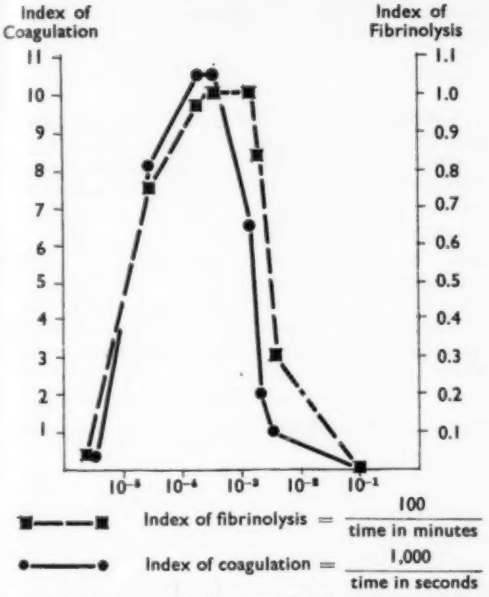

FIG. 4.—The influence of calcium concentration on the coagulation time and on the time of fibrinolysis in euglobulins.

fibrinogen, factor VII, and Christmas factor are considerably reduced. Euglobulins contain only traces of antiplasmin.

Figs. 3 and 4 show some essential features of euglobulin activation. Fig. 3 shows that Ca^{++} activation differs from streptokinase activation in that the Ca^{++} activation has released more trichloracetic acid-soluble tyrosine compounds at the time of clot lysis. Streptokinase-induced fibrinolysis occurs earlier and is associated with the release of less tyrosine.

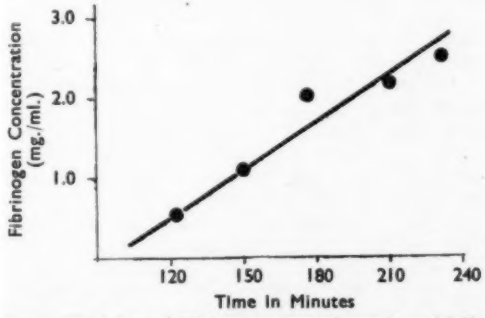

FIG. 5.—The influence of fibrinogen concentration on the euglobulin fibrinolysis time.

TABLE III
RELATION BETWEEN PLASMA FIBRINOGEN LEVEL AND EUGLOBULIN LYSIS TIME

Fibrinogen (mg. ml.)	Lysis Time in Minutes												
	0–30	31–60	61–90	91–120	121–150	151–180	181–210	211–240	241–270	271–300	301–330	331–360	361–390	> 390
0–1					1			1	1					
1·1–2				5	10	2	3	3	2	2	1			
2·1–3		1	3	8	1	4	4	5	2	2	1	1	1	
3·1–4			2	1	5	5	2	6	3	2	1	1		
4·1–5			2		2	1	4	4	4	1	1	2		
5·1–6					2	1			2					
6·1–7														

Fig. 4 presents the interrelationship between clotting and fibrinolysis in euglobulins. It can be seen that optimum clotting ability and maximum fibrinolysis occur at approximately the same calcium concentrations.

To investigate the effect of the fibrinogen concentration in the euglobulin fraction on the lysis time, increasing amounts of fibrinogen were added to the euglobulin fraction, but there is no correlation between the fibrinogen concentration in plasma and the euglobulin fibrinolysis time; this is shown (Table III) in a group of normal subjects and in patients with various conditions.

There is also no correlation between the fibrinolytic activity of whole plasma and fibrinolysis time in the euglobulin fraction as shown in Fig. 6.

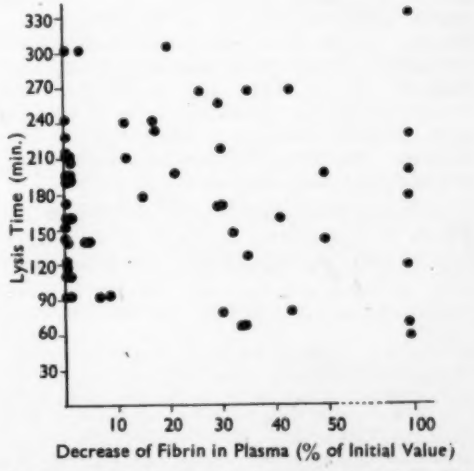

FIG. 6.—Relation between fibrinolysis in euglobulins and in whole plasma.

Discussion

The biochemical data presented show that there are essential differences between fibrinolysis in euglobulins and whole plasma. Ca^{++} activation as done by the euglobulin method cannot be considered as physiological. Rechnic, Siwinska, and Czerwinska (1957) and Buluk and Januszko (1957) have shown that the mechanism of activation of fibrinolysis in euglobulins is connected with a decrease of antiplasmin during thrombinogenesis. For this and other reasons euglobulin lysis does not reflect the real fibrinolytic potential in the organism. It was shown in confirmation that lysis time in the euglobulin fraction differs from fibrinolysis in whole plasma.

In some diseases, e.g., in cirrhosis of the liver, it is possible to show enhancement of fibrinolysis by whole plasma and by Dausset's method (Dausset, Paraf, Bergerot-Blondel, and Caroli, 1956) while the euglobulin method shows only insignificant changes.

Nevertheless, as seen from the material presented, the euglobulin method can be helpful in discovering disturbances in the fibrinolytic system, e.g., after the injection of adrenaline the euglobulin lysis time is always shortened. It seems that the lengthening of fibrinolysis time in chronic lung diseases can also be demonstrated adequately by this method (Kopeć *et al.*, 1952).

To some extent the value of the euglobulin method lies in the possibility of comparing large groups of patients and of drawing some conclusions about the nature of the fibrinolytic system. Various workers have used this method to show the activation of fibrinolysis in patients with tumours (Taper, 1956) and in patients with insulin hypoglycaemias (Hintz, 1956) and also to show diurnal variations of fibrinolytic activity (Kaniak, Siwinska, Krakowska - Rechnicowa, Iwankiewicz, and Kornaszewski, 1957).

Niewiarowski and Panasewicz (1954) used this method to demonstrate activation of the fibrinolytic system in heterogenic post-transfusional shock in animals and Niewiarowska (1957) in a study of physiological hypothermia.

Summary

The authors present the results of several years' experience of the application of the euglobulin method to determine fibrinolysis in human plasma. The results suggest that this method is valuable for clinical purposes, especially for the comparative assay of fibrinolytic systems in large groups of patients. The recovery of several factors of the clotting and fibrinolytic system in the euglobulin fraction is presented. Different types of activation of fibrinolysis in euglobulins are described. The biochemical background of the method is briefly discussed.

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MERCURY IN BIOLOGICAL MATERIALS

BY

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The present communication is concerned with the determination of mercury in biological materials and its normal excretion in humans.

As a preliminary step in the determination of mercury in biological materials, destruction of the organic matter is essential. Mercury has a high degree of volatility and hence precautions are necessary to prevent losses during this process. There have been many communications on this subject (cf. Sandell, 1950), but the simpler procedures are mostly applicable to urine only. Abbott and Johnson (1957) have described a relatively simple procedure for the determination of mercury in apples. The material is heated with a mixture of sulphuric and nitric acids in the presence of selenium in a special reflux apparatus. Oxidation is completed by the addition of hydrogen peroxide. This procedure has been found to be applicable not only to urine but to faeces and tissues. The hydrogen peroxide has been replaced by potassium permanganate, since a lower blank value can thus be obtained.

The most convenient method for the colorimetric determination of mercury is with dithizone using a reversion technique (cf. Irving, Risdon, and Andrew, 1949). Of the reversion techniques described, that using sodium thiosulphate in an acid medium (Abbott and Johnson, 1957) has been used. The use of a reversion technique involves two photometric measurements: (*a*) a solution containing unchanged dithizone and the metal dithizonate; and (*b*) the previous solution after reversion, now containing the original unchanged dithizone and dithizone derived from the metal dithizonate. The difference between these two measurements is representative of the quantity of metal dithizonate present in (*a*). Assessment may be made by taking readings at the wavelength of maximum absorption of dithizone, i.e., 620 mμ, or of the metal dithizonate, i.e., 430 mμ for mercury dithizonate. The differences are much greater at 620 mμ than at 430 mμ. At 620 mμ the differences (*b*–*a*) are positive, while at 430 mμ they are negative. It was therefore decided that it was preferable to carry out photometric measurements at 620 mμ rather than at 430 mμ. The instrument used in the present investigation was the "unicam" diffraction grating spectrophotometer S.P. 350.

The method appears to be specific for mercury. The effect of copper and silver, two metals that occur in trace amounts in the human diet and might cause interference, has been examined. It has been found that these metals are not included in the final result. Non-interference from silver depends partly on the presence of excess of chloride ions, viz., hydroxylamine hydrochloride.

The following are the details of the method.

Method

Reagents.—The following are required:

(1) Concentrated sulphuric acid (Analar).

(2) Concentrated nitric acid (Analar).

(3) 5% (w/v) potassium permanganate solution.

(4) 20% (w/v) hydroxylamine hydrochloride solution.

(5) Freshly prepared 1.5% (w/v) sodium thiosulphate solution.

(6) Freshly prepared dithizone, 4 mg./litre in chloroform.

(7) 0.1 N hydrochloric acid.

(8) Selenium powder.

(9) Standard mercury solution, 1 mg. Hg/ml.—0.135 g. $HgCl_2$ in 100 ml. of 0.1 N hydrochloric acid.

Procedure for Urine.—A 500-ml. conical flask is fitted with a 12-in. air condenser, and above this is located a water-cooled reflux condenser. The equipment is of all-glass construction (Quickfit and Quartz, Ltd.). The two digestions described below are carried out in the apparatus with both condensers *in situ*.

Into the conical flask are measured 100 ml. of urine, 15 ml. of concentrated sulphuric acid, 5 ml. of nitric acid, and 0.1 g. of selenium powder. The mixture is heated to boiling and maintained at this temperature for two hours. The mixture is cooled and then 10 ml. of 5% potassium permanganate solution added. The mixture is boiled for a further half hour. After cooling, the mixture is diluted to

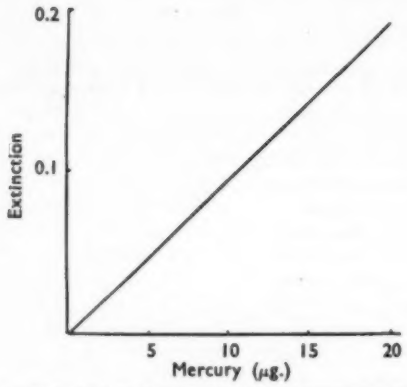

FIG. 1.—Determination of mercury by dithizone (reversion technique) in a "unicam" spectrophotometer S.P. 350—620 mμ.

250 ml. with water and filtered through an acid-washed filter paper.

Into a 500 ml. separating funnel are introduced 125 ml. of filtrate and 20 ml. of hydroxylamine reagent. After mixing, 30 ml. of dithizone reagent is added and the whole shaken vigorously for two minutes. A sample of the chloroform extract is separated and the extinction read against chloroform at 620 mμ (A). The complete chloroform extract is now transferred to a separating funnel containing 50 ml. of 0.1 N hydrochloric acid. Sodium thiosulphate solution, 2 ml., is added and the mixture shaken vigorously for two minutes to effect reversion. The chloroform solution is separated and the extinction read against chloroform at 620 mμ (B).

The difference between readings (B) and (A) is a measure of the mercury content which can be evaluated by reference to standards.

Standardization.—Mercury, 5, 10, 15, and 20 μg., in the form of mercuric chloride in 100 ml. of water, is examined using the complete procedure described above. Within this range the calibration curve is a straight line running through the origin (Fig. 1).

Blanks.—The complete procedure is carried out using 100 ml. of water.

Procedure with Faeces.—Faeces are emulsified with water and made up to a convenient volume, e.g., a 24-hour output may be emulsified up to 250 ml. with water.

Faecal suspension, 20 ml., is diluted to 100 ml. with water and then examined as described for urine.

Procedure with Tissues.—Tissues may be examined in the same manner as faeces.

In the event of the sample containing more than 20 μg. of mercury, then a smaller aliquot than that suggested above should be used. This should be diluted to 125 ml. with blank solution before examination with dithizone.

The first dithizone extract may be conveniently divided into two equal parts, and one part only subjected to reversion. By reading (B) directly against (A), only one photometric reading is required.

Results

The Recovery of Mercury.—Mercury added to urine (5 to 40 μg./100 ml.) could be recovered with an accuracy of 91 to 98% (see Table I).

Mercury added to faecal suspension (10 to 40 μg./10 ml.) could be recovered with an accuracy of 88 to 98% (see Table I).

Reproducibility of Results.—The results shown in Table II and Table III indicate that reproducible results may be obtained within approximately ±5%.

TABLE I

RECOVERY OF MERCURY ADDED TO URINE (100 ML.) OR TO FAECAL SUSPENSION (20 ML.)

Test No.	Initial Content (μg.)	Mercury Added (μg.)	Mercury Determined (μg.)	Mercury Recovered (μg.)	% Recovered
	Urine				
		5	4·5		90
1	Not detectable	10	9·5		95
2	,,	20	19·0		95
3	,,	30	27·5		92
4	,,	40	39·0		98
		5	4·5		90
5	,,	10	9·5		95
6	,,	20	19·0		95
7	,,	30	29·5		98
8	,,	40	36·5		91
	Faeces				
1	9	10	18·0	9·0	90
2	9	20	28·5	19·5	98
3	9	30	36·0	27	90
4	9	40	46·5	37·5	94
5	6·5	10	15·5	9·0	90
6	6·5	20	24·0	17·5	88
7	6·5	30	34·0	27·5	92
8	6·5	40	45·0	38·5	96

TABLE II

REPRODUCIBILITY OF RESULTS IN FAECAL SUSPENSIONS (20 ML.)

Sample	Micrograms Hg		
Examination:	1	2	3
A	5·0	5·5	4·5
B	9·0	9·0	8·5
C	12·0	12·5	11·5

TABLE III

DETERMINATIONS OF MERCURY IN PRESENCE OF COPPER AND SILVER

Mercury Used (μg.)	Mercury Determined (μg.)
In presence of 100 μg. *copper*	
10	9·5, 10·5
20	18·5, 20·5
40	39·0, 41·0
In presence of 100 μg. *silver*	
10	10·0, 10·5
20	19·5, 20·5
40	39·0, 40·5

Copper and Silver.—Both copper and silver will under appropriate conditions form coloured dithizonates in acid solution. The results indicated in Table III show that there is no interference in the determination of mercury under the conditions described in this paper.

Normal Excretion.—The mercury content of the urine from 24 humans without a history of abnormal exposure to mercury has been examined. Actually the content was so low as to be indeterminable by the present procedure. It was concluded that the daily excretion of mercury by the normal is less than 5 μg./day.

Faeces were examined in 24 cases without a history of abnormal exposure to mercury. All were collections over a period of four or five days. The mercury content was assessed at 55 to 180 (average 90) μg./day (see Table IV).

TABLE IV

EXCRETION OF MERCURY IN URINE AND FAECES OF NORMAL SUBJECTS

Specimen No.	Mercury (μg./day)	Specimen No.	Mercury (μg./day)
Urine			
24 cases—indeterminable—less than 5 μg. Hg/day			
Faeces			
1	55	13	85
2	95	14	125
3	60	15	145
4	180	16	75
5	125	17	85
6	75	18	80
7	85	19	95
8	140	20	65
9	90	21	95
10	60	22	70
11	65	23	80
12	95	24	75

Mean: 90 μg./day.
Standard deviation: 30·2

Discussion

Information regarding the normal excretion is essential if an assessment of abnormal exposure to the metal is to be made. Monier-Williams (1949) has summarized much of the available data.

Buckell, Hunter, Milton, and Perry (1946) have reported normal urinary excretion of mercury of 5 to 90 μg./day whereas the figures reported by Monier-Williams are of the order of 1 μg./day. The latter is indeterminable by the usual methods. The results reported in this paper are more in agreement with those of Monier-Williams.

According to Monier-Williams the normal intake of mercury is of the order of 20 μg./day. The analysis of individual foodstuffs is laborious, but one author (Tompsett, 1956) has indicated that a fair estimate of the alimentary intake of trace heavy metals may be made from an examination of faeces since urinary excretions and retentions are usually minute in comparison. From the results presented in this paper it would appear that the normal intake of mercury in the diet is of the order of 100 μg./day. Assuming that retentions are minimal it would appear from urinary examinations that absorption from the alimentary tract is minute.

An examination of urine does not necessarily present direct evidence of abnormal exposure to a heavy metal especially if exposure has ceased for some time. The use of chelating agents (cf. Leckie and Tompsett, 1958) could prove of value in such problems.

Summary

A method has been described for the determination of mercury in biological materials (urine, faeces, and tissues).

The normal excretion of mercury in urine was found to be less than 5 μg./day, and in faeces 55 to 180 μg. Hg/day.

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J. clin. Path. (1959), **12**, 222.

CHANGES IN BUCCAL CELLS IN THE ANAEMIAS

BY

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Morphological abnormalities of the haemopoietic cells in pernicious anaemia have been well known for many years, but it is only recently that attention has been drawn to abnormalities in the epithelial cells. Graham and Rheault (1954) studied the buccal epithelial cells in gastric washings and showed that there was general enlargement of the cytoplasm and of the nucleus, the presence of some cells with much enlarged nuclei, and an increase in the number of cells with two or more nuclei. The nucleus may be hyperchromatic, with unevenly distributed chromatin and a prominent nucleolus, and have an irregular membrane.

Massey and Klayman (1955) and Rubin (1955, 1956) confirmed these findings, and, with Gardner (1956), found that these changes are also present in megaloblastic anaemia not due to vitamin B_{12} deficiency; however, they are not completely specific for megaloblastic anaemia. Changes in various other epithelia from cases with megaloblastic anaemia have been reported by several authors (Boddington and Spriggs, 1959).

Górz-Kardaszewicz (1956), Boen (1957), and Farrant (1958) measured abnormal cells from the mouth in cases of pernicious anaemia and confirmed that the nucleus in the buccal cells is abnormally enlarged but this is reversible with treatment.

Scarcely any mention has been made of the condition of the tongue in the cases so far examined, and it therefore seems highly relevant to discover whether the cytological changes in buccal cells are dependent upon atrophy of the papillae or on the megaloblastic anaemia. The present study has been made with this end in view.

Material and Methods

Initially tongue scrapings taken with a glass slide were used for this study, but specimens of saliva later proved more satisfactory. In some cases both types of specimen were examined (Boddington and Spriggs, 1959). Comparison of the average diameters of the cytoplasm and of the nucleus in specimens taken by the two methods in the same patient showed little variation in the general pattern. In all cases the nuclear diameter in saliva was greater than in the tongue smear and in the majority the difference was a significant one.

Saliva also made possible a direct comparison with the buccal cells seen in gastric washings. In 10 cases the average diameters in saliva showed close agreement with those in gastric washings taken at the same time. The tendency was towards smaller cells and nuclei in the gastric washings, but not in all cases.

In this paper only the results obtained from saliva are reported. Specimens were taken from 59 patients of whom 18 were diagnosed as pernicious anaemia by the usual criteria, 11 as other megaloblastic anaemias (due to pregnancy, five cases, steatorrhoea, two cases, total gastrectomy, one case, nutritional deficiency, one case, carcinomatosis, one case, and phenobarbitone treatment for epilepsy, one case). Twenty had iron-deficiency anaemia, and 10 cases had abnormal tongues not associated with anaemia. Twenty-three patients had papillated tongues and in the rest the papillae were atrophied to a greater or lesser degree. Specimens from 10 people of different age groups were used as controls.

Specimens were collected by asking the patient to spit several times into a container. The saliva was centrifuged and two films made from the deposit either with a wire loop or by spreading between the two slides; these were immediately "wet-fixed" in ether-alcohol or ethyl alcohol containing 3% acetic acid for staining by the Papanicolaou technique.

The slides were examined through the 1/12 oil-immersion objective, and the mean diameter of 100 cells and their nuclei in each case was determined by measuring both the long and the short diameters with an eyepiece micrometer. Some selection was necessary to avoid those cells with folded cytoplasm, unusually distorted nuclei, and those in tight collections. As far as possible the measurable cells were consecutive. The mean diameter of these measurements was calculated for each case and this is the "mean diameter" referred to below. In addition, percentages of abnormally enlarged nuclei (greater than 14 μ in diameter) and of binucleate cells were calculated from counts of 1,000 cells.

Results

Measurement of Diameters.—These are set out in the Table and Fig. 4.

TABLE
AVERAGE DIAMETERS OF BUCCAL SQUAMOUS CELLS

Disease Group	No. of Cases	Average Cytoplasmic Diameter and Standard Error (μ)	Average Nuclear Diameter and Standard Error (μ)
Tongue papillated :			
Normal control	10	50·11±1·17	8·64±0·17
Pernicious anaemia ..	5	51·65±1·17	8·66±0·08
Other megaloblastic anaemias	4	51·48±1·37	8·93±0·21
All megaloblastic anaemias	*9*	*51·57±0·87*	*8·78±0·08*
Iron-deficiency anaemia ..	10	46·46±0·94	8·94±0·11
Other cases with abnormal tongues	4	43·86±2·62	8·83±0·28
Tongue depapillated :			
Pernicious anaemia ..	13	49·32±1·49	9·92±0·27
Other megaloblastic anaemias	7	47·33±2·14	10·28±0·15
All megaloblastic anaemias	*20*	*48·77±1·22*	*10·10±0·19*
Iron-deficiency anaemia ..	10	48·55±1·33	9·41±0·16
Other cases with abnormal tongues	6	44·96±1·27	9·39±0·16

Normal Buccal Cells.—Buccal squamous cells from a normal healthy person have a cytoplasmic diameter from 20 μ to 80 μ, with a mean of about 50 μ. The nucleus is small and oval with a fine regular chromatin pattern (except when pyknotic) and a diameter varying from 5 μ to 13 μ, with a mean of about 8.6 μ. Occasional binucleate cells are present. The cytoplasm frequently contains kerato-hyaline granules and often there is a perinuclear halo. The appearance of these cells is shown in Fig. 1.

Cells from Cases of Megaloblastic Anaemia.—Many of the buccal cells from cases of megaloblastic anaemia have a normal appearance apart from the slight increase in nuclear diameter.

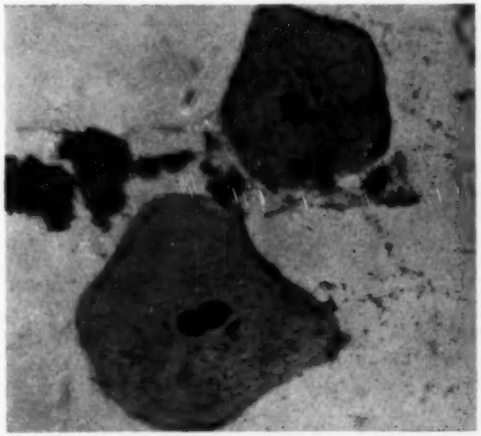

FIG. 1.—Normal buccal squamous cells. The lower one is binucleate and the other contains kerato-hyaline granules. × 400.

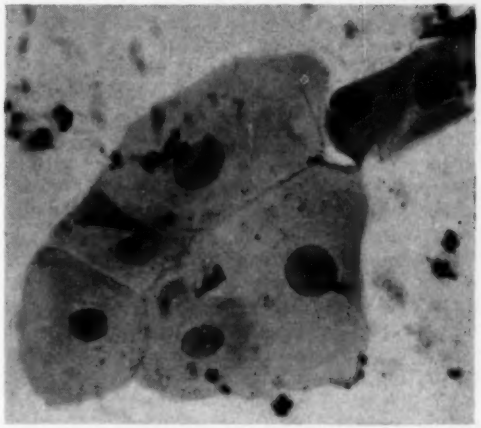

FIG. 2.—Buccal cells from a case of pernicious anaemia with a smooth tongue. All the nuclei are larger than normal and the two largest cells have "giant" nuclei, i.e., greater than 14 μ in diameter. × 400.

Occasional nuclei, however, are hyperchromatic with prominent nucleoli and a serrated nuclear membrane, but are not usually much enlarged. The greatly enlarged (giant) nuclei are commonly vesicular and round with a regular nuclear border (Fig. 2). The cytoplasmic diameter in these cells is rarely very large and is commonly even smaller than normal.

Measurements of cytoplasmic diameters showed close agreement with those of the normal series. The cases with normal tongues tended to have slightly larger diameters than normal, while in the group with depapillated tongues they were rather smaller (see Table). In only one case was there any considerable increase in size. In six cases examined 10 to 15 days after treatment with vitamin B_{12} there was a decrease in diameter in five, while in the sixth case the tongue remained smooth in spite of treatment.

The nuclear diameters, on the other hand, were essentially normal in the cases with papillated tongues, but those with smooth tongues had much larger nuclei; the difference between the averages for this group (see Table) and that of the controls was highly significant ($P = <0.001$).

One patient with a normal tongue showed a high mean diameter, whereas two of them with smooth tongues had diameters within normal limits (Fig. 4). Five of the six patients examined after treatment showed the expected reversion towards normal; two had subnormal diameters after 10 and 15 days respectively.

Cells from Cases of Iron-deficiency Anaemia.—Changes in the buccal cells in iron-deficiency anaemia have not so far been reported. This study has shown that the cytoplasmic diameters in the 20 cases examined tended to be smaller than normal especially in 10 cases with papillated tongues (see Table). The difference between the average for this group of 10 and that of the normal controls is a significant one at the 5% level.

The mean nuclear diameters of the 10 cases with normal tongues were within normal limits although in two cases there was some enlargement. On the other hand, most of the cases with atrophy of the papillae showed nuclear enlargement (Fig. 4). In some cases the diameters were comparable to those seen in megaloblastic anaemia (Fig. 3) and the average for this group showed a statistically significant increase over normal ($P = <0.05$).

Cases with Abnormal Tongues not Associated with Anaemia.—The 10 cases with abnormal or smooth tongues in the absence of anaemia in which buccal cells were measured all had normal haemoglobin values. (Six had completely depapillated tongues, two had thrush, one had leukoplakia of the tongue, and one had a painful tongue normal in appearance of unknown aetiology.)

In all but one of this miscellaneous group there was a smaller cytoplasmic diameter than normal and both the groups (see Table) showed a significantly smaller average diameter at the 5% level.

The nuclear diameters were within normal limits in three of the cases without epithelial atrophy of the tongue, and in two of those with a depapillated tongue (Fig. 4). The group without atrophy showed an average diameter which was essentially normal, whilst the group with smooth tongues (see Table) had a diameter which was significantly higher than normal ($P = <0.05$).

Percentages of Nuclear Abnormalities.—Fig. 5 illustrates these in the patients and controls.

The most readily observed changes and those which are most easily measured are the greatly enlarged (giant) nuclei and the binucleate cells. These appeared to be more numerous in megaloblastic anaemia than in other conditions, and in order to verify this supposition the percentages of nuclei greater than 14 μ in mean diameter and the cells with two or more nuclei in counts of 1,000 cells were found for each case. Fig. 5 shows the mean values for each group.

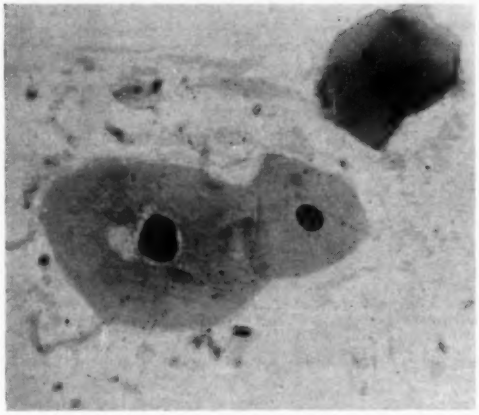

FIG. 3.—Buccal cells from a case of iron-deficiency anaemia with a smooth tongue. The largest cell has a "giant" nucleus which is also hyperchromatic with three prominent nucleoli and an irregular border. × 400.

Nuclei Greater than 14 μ in Diameter.—No nuclei of this size were seen in specimens from normal persons.

"Giant" nuclei were found in all cases of megaloblastic anaemia with smooth tongues. Two cases with near normal diameters showed quite frequent giant nuclei and small percentages were also found in five of the cases with papillated tongues, four of them having normal mean diameters.

None of the cases with iron-deficiency anaemia having normal tongues had abnormally large nuclei, but all but two of the group with smooth tongues showed them (Fig. 3).

Of the 10 cases without anaemia five with increased nuclear diameters also showed "giant" nuclei.

"Giant" nuclei, therefore, were present in the majority of cases with smooth tongues and in half of the cases of megaloblastic anaemia with normal tongues. Whilst many of the cases of megaloblastic anaemia had a high percentage of large nuclei (over 5%) and the mean values (Fig. 5) show a striking difference from the means of the other groups, there is some overlap between the groups and no specific diagnostic value can be attached to the "giant" nuclei alone.

Multinucleation.—Cells with more than two nuclei were rarely seen. Graham and Rheault (1954) and Górz-Kardaszewicz (1956) have reported cells with five and nine nuclei respectively, although the photomicrographs of these

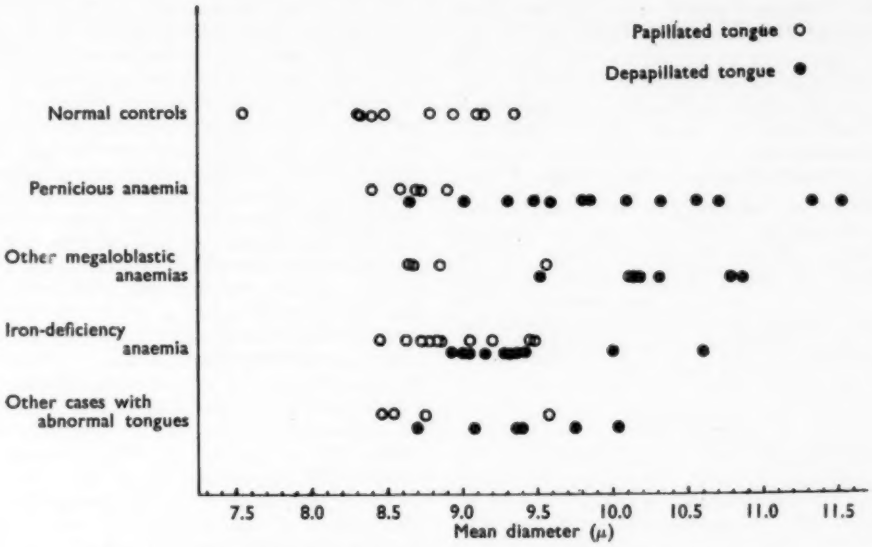

FIG. 4.—The mean nuclear diameters for each case examined.

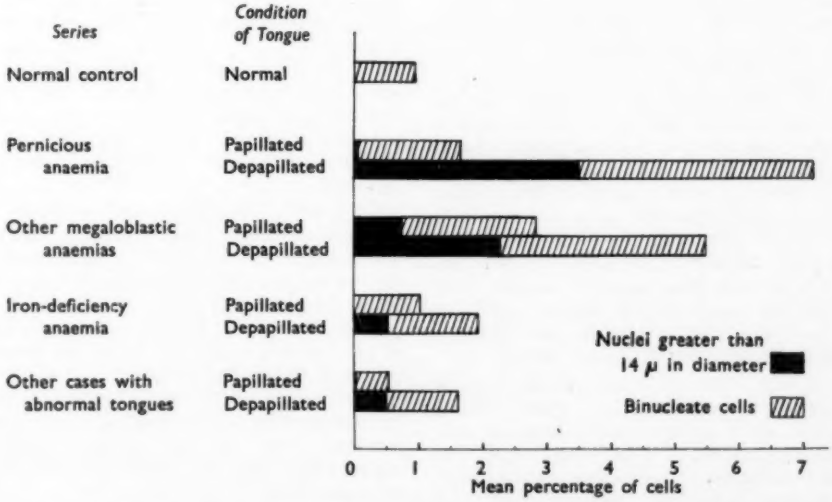

FIG. 5.—The mean percentage values of nuclei greater than 14 μ in diameter and of binucleate cells in each group of cases.

suggest that they may in fact have been cell clusters. An acceptable example has been published by Massey and Klayman (1955).

The percentages of binucleate cells calculated from counts of 1,000 cells again showed them to be more numerous in cases with megaloblastic anaemia although not necessarily correlated with the "giant" nuclei (Fig. 5). There was still some overlap between the normals and the other groups.

Other Abnormalities.—Nuclear abnormalities such as hyperchromatism with unevenly distributed chromatin and irregular nuclear membrane are qualitative, and interpretation will vary from one observer to another. Such abnormalities may be more frequent in cases with megaloblastic anaemia, but are not always present and do not appear to be specific.

Conclusions

Graham and Rheault's (1954) initial observation, later confirmed by Boen (1957), of the enlargement of the cytoplasmic diameters in buccal squamous cells in cases with pernicious anaemia was not confirmed in this investigation.

The cells in pernicious anaemia were not much changed in size from those in normal persons and tended to be smaller in those cases with atrophy of the lingual epithelium. This tendency towards a smaller cytoplasmic size was more apparent in cases with iron-deficiency anaemia and certain other diseases. In addition the cytoplasmic diameter decreased still further in pernicious anaemia after treatment.

We have confirmed that in many cases of megaloblastic anaemia the buccal cell nucleus is abnormally large and that there are other nuclear abnormalities. In the majority of cases, however, these abnormalities were associated with atrophy of the lingual epithelium and were also present in cases with certain other diseases where a smooth tongue was present. Farrant (1958) has described one such case without anaemia, and lingual atrophy may account for the six cases which did not have megaloblastic anaemia described by Massey and Klayman (1955).

Cases in our series of megaloblastic anaemia with normal tongues did not show nuclear enlargement, and it was not invariable in the cases with smooth tongues. Boen (1957) has also described three cases of pernicious anaemia with normal nuclear diameters. In such cases there may be other nuclear abnormalities, also non-specific, but usually much more marked in megaloblastic anaemia.

Gardner (1956) described nuclear fragmentation and nuclear haloes as additional abnormalities in tropical sprue. Nuclear fragments in the cytoplasm were never seen in our preparations, and Gardner may have been observing kerato-hyaline granules. Percentages of cells containing these vary considerably in each specimen, and we have found no correlation with the disease or the state of the tongue (thus confirming Górz-Kardaszewicz, 1956). Perinuclear haloes are not specific for megaloblastic anaemia and are a normal feature of buccal cells (Ziskin and Moulton, 1948). Certain cases did show extreme examples of this phenomenon, and our case of tropical sprue was one of them.

Whilst concluding that percentage values of such nuclear abnormalities as gigantism and binucleation are not an adequate diagnostic test for megaloblastic anaemia in all cases, these may be high enough to assist in the diagnosis. Boen, Molhuysen, and Steenbergen (1958) have demonstrated such an instance.

It is evident from our series that the abnormal enlargement of the buccal cell nucleus is not directly correlated with the smoothness of the tongue. This is illustrated by the finding of this abnormal change in cells from cheek scrapings (Górz-Kardaszewicz, 1956; Farrant, 1958). It seems that these abnormalities appear at a stage in the deficiency state when the tongue also (in most cases) becomes clinically abnormal. No attempt was made to classify the degree of lingual atrophy, but it is of interest that most of the cases in the "smooth" group with normal or near-normal nuclear diameters did not have completely depapillated tongues. A method of indexing the degree of depapillation, such as that of DiPalma (1946), would possibly give a closer correlation.

It appears, therefore, that in megaloblastic anaemia the abnormality in cellular metabolism, whose nature is still not fully understood, at the same time causes the megaloblastic transformation in the red cell precursors, the gigantism of the granular leucocytes, and the nuclear abnormalities of the epithelial cells (of which the buccal cells are the most strikingly affected). The enlargement of the nucleus of the buccal squamous cell may be produced by other metabolic defects such as iron-deficiency, but the present methods of examination cannot distinguish between the different causes.

Summary

Mean cytoplasmic and nuclear diameters of salivary squamous cells were measured in 69

persons. Eighteen of these had pernicious anaemia, 11 had other megaloblastic anaemias, 20 had iron-deficiency anaemia, 10 had abnormal tongues not associated with anaemia, and 10 were normal controls. Thirty-six of the patients had smooth tongues.

The cytoplasmic diameters of buccal cells from cases of pernicious anaemia were not enlarged but tended to be smaller than in normal healthy persons. In other megaloblastic anaemias, iron-deficiency anaemia, and in some other diseases not associated with anaemia but having abnormal tongues, the cytoplasmic diameter also appeared to be abnormally small.

The nuclei of buccal cells showed a distinctly enlarged diameter in those cases of megaloblastic anaemia in which there was lingual atrophy. Cases of iron-deficiency anaemia also sometimes showed this nuclear enlargement in the presence of a smooth tongue, and a high nuclear diameter is by no means specific for any particular cause of lingual atrophy. However, the highest figures were found in pernicious anaemia.

Specific nuclear abnormalities such as "giant" nuclei and binucleation were more common in megaloblastic anaemia than in other disease groups with smooth tongues. A small percentage of abnormal nuclei could be seen in cases of megaloblastic anaemia with normal tongues. These abnormalities are not a specific diagnostic test for the megaloblastic anaemias but may assist diagnosis when they are present to a marked degree.

My thanks are due to the medical staff of the United Oxford Hospitals for providing me with specimens and details of patients under their care; to Mrs. D. Jackson for her assistance with the photography; and to Mr. N. T. J. Bailey for his advice on the statistics.

I am also grateful to Dr. A. H. T. Robb-Smith for correcting the manuscript and in particular to Dr A. I. Spriggs for all the advice and help given during this investigation.

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*As it is only available in Polish the following summary of the publication by Górz-Kardaszewicz (1956) is given.

From 10 to 15 cheek scrapings at three- to four-day intervals were taken from 1 cm. below the right parotid duct in each patient, and examined in wet-fixed films stained with haematoxylin and eosin. Percentages of abnormal cells were calculated in each of 32 patients with anaemia (22 pernicious anaemia) and in 10 normal males and 10 normal females. The abnormalities counted were large cells with normal nuclei, large nuclei in normal cells, multinucleate cells, cells containing cytoplasmic granules, and anucleate cells.

The results were tabulated and showed about 10% of abnormal cells in pernicious anaemia. Cells with a total diameter above 91 μ accounted for 4% of this increase, those with nuclei above 16 μ in diameter 2%, and binucleate cells 4%. The abnormal enlargement persisted in a smaller percentage of cells in 16 cases after treatment. The number of cells with granules and anucleate cells was not abnormally high.

Górz-Kardaszewicz postulates that the same factor controls both the changes in the epithelium and in the marrow because gigantism and multinucleation can be found in cells obtained from all accessible regions of the body, but no data for this hypothesis are given.

J. clin. Path. (1959), **12**, 228.

THE EPITHELIAL CELLS IN MEGALOBLASTIC ANAEMIAS

BY

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The earliest cytological abnormalities to be recognized in pernicious anaemia were, of course, those of the blood. Apart from the various changes seen in the mature red cells, circulating nucleated precursors showed sufficiently striking alterations for Ehrlich to give them the separate name of megaloblast (Ehrlich, 1880). More recently, the granular leukocytes were found to be hypersegmented, and after the introduction of marrow puncture the gigantism of the immature granular cells became an equally familiar feature of this group of diseases.

In view of the association of anaemia, achlorhydria, glossitis, prematurely white hair, and degeneration of the spinal cord, it was not surprising to learn that cytological abnormalities can be found outside the haemopoietic system. Rubin and Massey in 1953 briefly reported the finding of enlarged nuclei in gastric mucosal cells from gastric washings of patients with pernicious anaemia, and since they were all in therapeutic remission this was presumably a feature of the permanent gastric atrophy. Graham and Rheault (1954) then described enlargement of both nucleus and cytoplasm of the squamous epithelial cells in gastric washings (cells presumably derived from swallowed saliva). This observation was supported by photomicrographs and measurements in 15 cases of pernicious anaemia in relapse, and has been confirmed by other observers ; it is rapidly reversed by vitamin B_{12} treatment. At the same time, Graham and Rheault claimed to have found that gastric columnar cells and macrophages from the same specimens also showed enlargement. The data on these were much less convincing, depending on a comparison of a single case with a single control ; and in the case of the macrophages there has been no subsequent confirmation. It was also mentioned that a similar abnormality had been seen in a vaginal smear, but no data were given. Graham (1957) has also stated that the same changes can be found in vaginal smears of young mice born of vitamin B_{12}-deficient mothers.

The enlargement of the nuclei of the buccal cells can best be studied in smears of saliva, and one of us (M. M. B.) began to measure these cells in order to determine whether the changes are specific for megaloblastic anaemias or simply related to glossitis and atrophy of the tongue. The results (Boddington, 1959) showed that similar changes are indeed found in the saliva of patients with smooth tongues due to iron-deficiency and other causes, although not to the same degree as in certain cases of megaloblastic anaemia ; the same changes were also sometimes found in patients with megaloblastic anaemia whose tongues were not smooth. Nuclear enlargement was not constantly found in megaloblastic anaemias, even in the presence of some lingual atrophy.

Similar studies have been reported by Górz-Kardaszewicz (1956), Boen (1957), and Farrant (1958), all confirming that the buccal cells in pernicious anaemia exhibit nuclear enlargement but without relating the changes to the state of the tongue, so that the specificity of the abnormality remains unproved.

Meanwhile, Rubin and the Chicago school (Massey and Rubin, 1954 ; Massey and Klayman, 1955 ; Rubin, 1955, 1956) and Gardner (1956), besides confirming Graham and Rheault's findings with respect to the squamous cells of gastric washings and extending them to other types of megaloblastic anaemia, made extensive observations on the gastric mucosal cells. It appears that only a small proportion of these cells are affected, and, while the most striking abnormalities are reversed by treatment, the presence of "bland" or "active" enlarged columnar cell nuclei may persist, and is presumably related to the irreversible gastric atrophy. Neither of these types is specific for megaloblastic anaemia (Rubin, 1955 ; Henning and

Witte, 1957). Grable, Zamcheck, Jankelson, and Shipp (1957) measured the nuclear diameters of gastric mucosal cells and found much higher mean values in pernicious anaemia than in other conditions apart from carcinoma.

It still remained to discover whether any other cells of the body are really affected apart from those of the mouth and stomach, and, if so, whether the changes are any more specific in other areas. We have therefore studied a series of 10 cases of megaloblastic anaemia, obtaining cytological material from a variety of different sites, and comparing the morphology of the cells before and after response to treatment.

Material

Of the 10 patients studied, eight had Addisonian pernicious anaemia (with low serum vitamin B_{12} and histamine-fast achlorhydria), one had megaloblastic anaemia of the puerperium, and one had megaloblastic anaemia with a serum vitamin B_{12} level of 28 $\mu\mu$g./ml. but normal gastric acidity, probably attributable to treatment of epilepsy with phenobarbitone. Six of the patients were women and four men. All had megaloblastic marrows.

In each case at or before the start of treatment smears were made of saliva, tongue scrapings, nasal and conjunctival swabs, urinary sediment, and, in women, vaginal aspirates. The same series was repeated nine to 17 days after beginning treatment (Table I); in all cases a reticulocyte response had occurred.

Gastric washings were also examined in eight of the cases (six before and two after treatment), but paired samples were not obtained. The findings from these will form part of another series to be published.

TABLE I

CLINICAL DETAILS OF SERIES

Case No.	Sex	Age	Diagnosis	Time of Taking Second Specimen (Days after Starting Treatment)	Sources of Abnormal Cells Found in First Specimens
1	F	75	Pernicious anaemia	14	Mouth, nose, urine, vagina
2	M	46	,, ,,	15	Mouth
3	M	51	,, ,,	9	None
4	F	80	,, ,,	16	Mouth, nose
5	M	70	,, ,,	16	None
6	M	55	,, ,,	10	Mouth
7	F	68	,, ,,	11	,,
8	F	36	Megaloblastic anaemia of puerperium	14	Mouth, nose, vagina
9	F	49	Pernicious anaemia	13	Mouth, vagina
10	F	74	Megaloblastic anaemia in epileptic on phenobarbitone	17	Mouth, urine, vagina

Methods

The methods used in preparing the smears were as follows:

Saliva.—The patient spat into a container until about 5 ml. saliva was obtained. This was centrifuged until the cells were deposited, and the deposit was distributed on a glass slide with a platinum loop. It was immediately fixed while still wet in alcohol containing 3% acetic acid for at least 15 minutes, then stained by the method of Papanicolaou.

Air-dried films were also made and stained with May-Grünwald and Giemsa exactly as used for blood films.

Tongue Scrapings.—The tongue was scraped to one side of the midline with the narrow edge of a glass slide, and the material spread on to another slide in the same manner used in making blood films. Fixation and staining were the same as described for saliva.

Nasal Swabs.—These were taken with a dry cotton-wool "throat swab." The swab was then gently stroked on to two slides. One was wet-fixed and stained by Papanicolaou's method, the other was rapidly dried in air and stained by May-Grünwald and Giemsa. Material was obtained from 20 normal persons as well as from the anaemic subjects.

Conjunctival Swabs.—These were taken with a cotton-wool swab near to the inner canthus, and immediately smeared on to a slide. As the material dried instantaneously, only the May-Grünwald stain was used. Slides from 20 control subjects were examined as well as those from the series.

Urine.—In three of the women catheter specimens were examined; in the rest, urine was used as passed. The specimen was centrifuged immediately after voiding, and the deposit was transferred on to slides with a platinum loop. Wet-fixed films were made and stained by Papanicolaou's method, and air-dried films were also made and stained with May-Grünwald and Giemsa.

Vaginal Smears.—These were made from aspirate obtained with a vaginal pipette, and were wet-fixed in alcohol containing 3% acetic acid and stained by Papanicolaou's method. In three post-menopausal cases nuclear measurements were made before and after treatment in order to demonstrate any significant change. For this purpose, 100 squamous epithelial cell nuclei in each slide were measured in two diameters, using an eyepiece micrometer and the 1/12 oil-immersion objective. The mean diameter was calculated for each nucleus, and hence the mean nuclear diameter for the set of 100.

Apart from the vaginal cell measurements referred to, and those of buccal cells being published on page 222, measurements were only made on isolated cells. The cells of conjunctival, nasal, and urinary smears are fewer, more variable and irregularly distributed, and we felt that the statistical method applied to these would give only a false sense of accuracy.

FIG. 1.—Nasal smear in untreated pernicious anaemia (Case 1). A nasal epithelial cell is shown with an enormously enlarged abnormal nucleus. Such cells are very uncommon and most of the nasal columnar cells show no abnormality. May-Grünwald Giemsa, × 700.

FIG. 2.—Nasal smear—same case as Fig. 1 after treatment. A group of nasal columnar cells showing normal morphology. May-Grünwald Giemsa. × 700.

Results

Saliva and Tongue Scrapings.—In all except Case 5 the tongues showed some degree of soreness or depapillation. In all but two patients (Cases 3 and 5) the buccal squamous cells showed nuclear enlargement. The changes in these cells will not be fully described here, as one of us has given an account of a larger series which includes some of the present cases (Boddington, 1959).

In the specimens obtained after response to treatment the nucleus was reduced in size in all cases where this was initially abnormal, but completely normal levels were not always reached. For instance, in Case 2, the mean nuclear diameter before treatment was 10.3 μ, and 15 days after beginning treatment it was 9.6 μ, which is still above the normal level; nuclei of diameter greater than 14 μ amounted to 4.8% before and 1.5% after treatment. (These have never been found in normal persons.)

Nasal Mucosal Smears.—In the controls, the nuclear diameter was usually about 14 μ in air-dried films and 12 μ in Papanicolaou films. There was, however, some variation between individuals, and a few large nuclei sometimes occurred in an otherwise uniform film. Nuclei with diameters up to 16.5 μ were occasionally encountered in air-dried films, where there is more variation owing to differences in spreading.

Of the series with megaloblastic anaemia, seven were considered to have nasal smears indistinguishable from normal. In three patients (Cases 1, 4, and 8) certain nasal columnar cells in the pre-treatment samples were judged to be abnormal (Figs. 1 and 2). These were rounded-up cells showing nuclear enlargement (up to 20 μ diameter in air-dried films and 15 μ in wet-fixed ones), and sometimes showing prominent nucleoli. In view of the variation in normals, and the small number of abnormal cells found in cases of pernicious anaemia, the evidence for any real recognizable change is meagre.

Conjunctival Smears.—Cells were scanty in films, but were fairly uniform in size and appearance. In no case was any difference noted between the cases of anaemia and the controls, or between the pre- and post-treatment samples.

Urine.—In non-catheter specimens from Cases 1, 4, and 7, most of the cells in the urinary deposit were similar to those of the vaginal smears. In Case 1, the first specimen contained some unusual cells probably from the urinary tract with a very

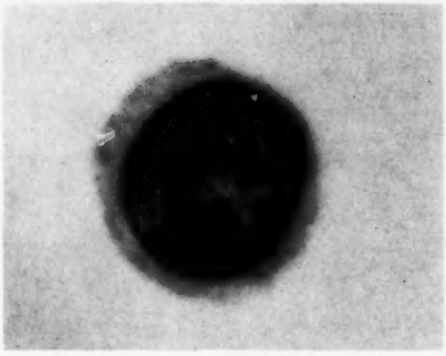

FIG. 3.—Urine deposit in untreated pernicious anaemia (Case 1). An epithelial cell showing nuclear enlargement and irregular hyperchromasia. Papanicolaou, × 2,300.

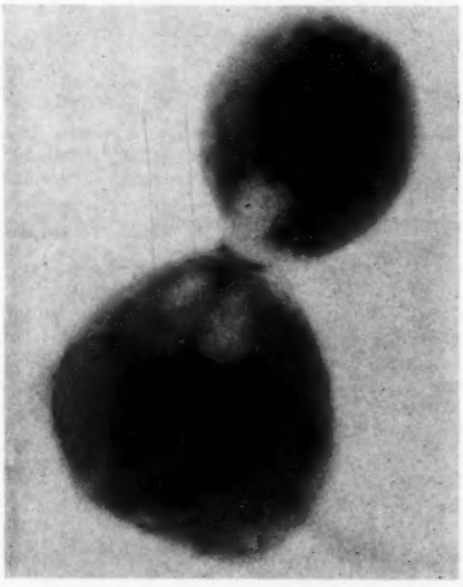

FIG. 4.—Urine deposit—same case as Fig. 3 after treatment. Two normal urinary epithelial cells at the same magnification for comparison. Papanicolaou, × 2,300.

high nucleo-cytoplasmic ratio and a "dotted" chromatin pattern (Fig. 3). These may be compared with normal cells in the second specimen (Fig. 4).

In the remaining three female cases, only catheter specimens were examined. In one of them, Case 10, the pre-treatment sample showed some cells with large nuclei (up to 19 μ diameter in a Papanicolaou film), a high nucleo-cytoplasmic ratio, hyperchromasia, and irregularities in nuclear outline, changes resembling those found in carcinoma of the bladder. The specimen obtained after treatment showed no abnormal cells.

The urine specimens from the four male cases showed no abnormalities.

Vaginal Smears.—Only three of the female cases provided comparable atrophic post-menopausal smears before and after treatment (Cases 1, 9, and 10). Owing to the hormonal changes which occur even in the elderly, it is difficult to be sure that any difference is due to the effect of treatment; these three, however, had no sign of cornification in the smears, and in Case 1 a hysterectomy and bilateral salpingo-oophorectomy had been done 18 years before.

Table II shows the figures obtained from nuclear measurements in these three cases. In Cases 1 and 10 there was a highly significant difference between the slides made before and after treatment. In Case 4 there was also a reduction in size, but the difference was not significant at the 5% level.

TABLE II

MEAN NUCLEAR DIAMETERS OF VAGINAL CELLS

	Mean Diameter and Standard Error (μ)	Standard Deviation	P
Case 1 Before B_{12} ..	10·11±0·22	2·17	<0·001
After B_{12} ..	8·49±0·15	1·45	
,, 4 Before B_{12} ..	8·63±0·16	1·62	Not significant
After B_{12} ..	8·20±0·22	2·17	
,, 10 Before B_{12} ..	9·48±0·13	1·34	<0·001
After B_{12} ..	8·23±0·13	1·26	

Morphologically, the vaginal cells in these three cases were of the "basal" type typical of post-menopausal atrophy. The nuclear enlargement in the pre-treatment samples was not associated with hyperchromasia; in Case 1 the nuclei had a pallid vesicular appearance (Figs. 5 and 6), while in Cases 4 and 10 they did not differ appreciably from normal. The nucleoli were not prominent. It is doubtful whether a diagnosis of megaloblastic anaemia could even be suspected from material so variable as vaginal smears, but the difference in measurements shown in these three cases, and particularly in Case 1, seems most likely to be a reflection of a morphological change due to the effect of vitamin B_{12} deficiency and its correction.

Gastric Washings.—These were examined in eight of the present series, six in relapse and two in remission. As they form part of a larger series,

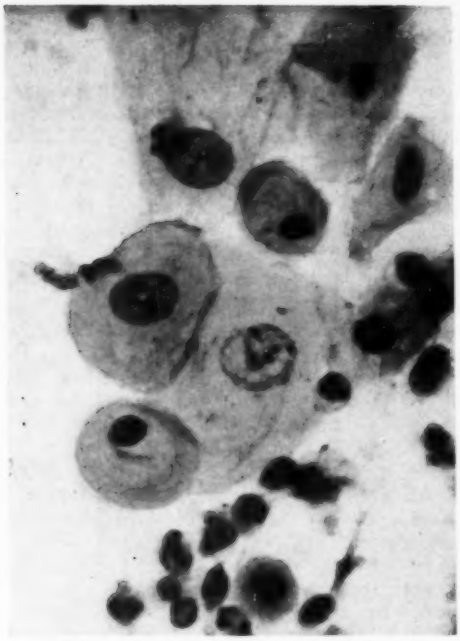

FIG. 5.—Vaginal smear in untreated pernicious anaemia (Case 1). A post-menopausal smear showing nuclear enlargement. Papanicolaou, × 700.

FIG. 6.—Vaginal smear—same case as Fig. 5 after treatment. Marked reduction of nuclear diameter compared with pre-treatment sample. Papanicolaou, × 700.

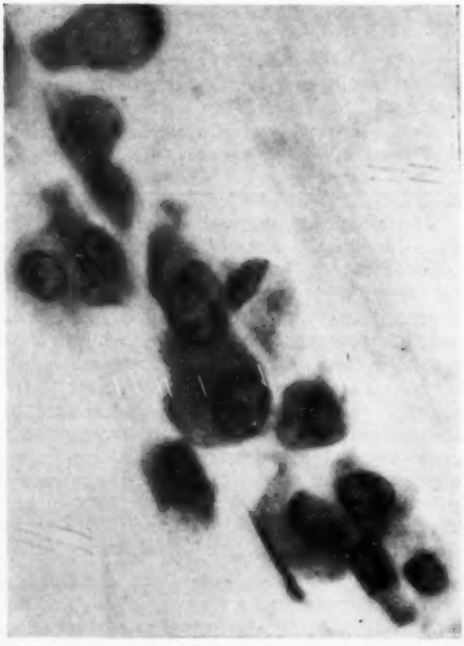

FIG. 7.—Sputum in untreated pernicious anaemia. A single collection of bronchial columnar cells was found showing striking nuclear enlargement. Papanicolaou, × 700.

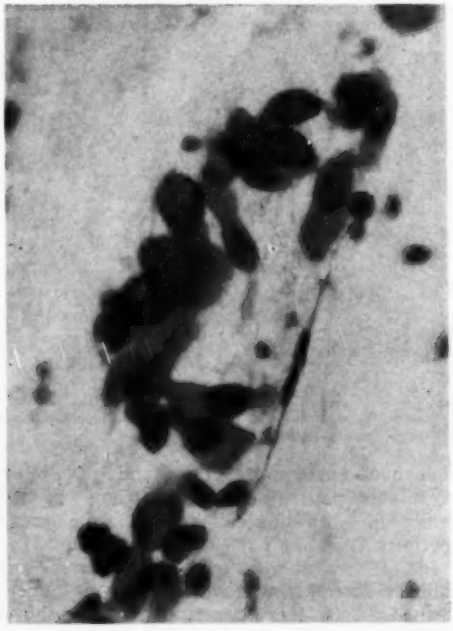

FIG. 8.—Normal sputum. A collection of bronchial columnar cells in normal sputum for comparison with Fig. 7. Papanicolaou, × 700.

the details will not be given here. Only one (Case 1) showed any appreciable abnormality of gastric mucosal cells apart from the intestinal metaplasia which is recognizable in some of the cases. The abnormal cells occurred in a single fragment, and showed anisocytosis and hyperchromasia; they corresponded apparently to Rubin's "active cells." The swallowed squamous epithelial cells did not differ appreciably from those of the saliva. No abnormality was noticed in the macrophages in any of the cases.

Bronchial Epithelium.—In a case of pernicious anaemia in relapse (not included in this series) specimens of sputum containing bronchial ciliated cells were examined. Some of these showed striking nuclear enlargement in Papanicolaou films, as well as prominent nuclei (Figs. 7 and 8). Only a small group of cells was affected, the remainder being morphologically normal.

Pleural Fluid.—Pleural fluid from one case of untreated pernicious anaemia not included in the present series was examined. The patient had tabes dorsalis, and although there was histamine-fast achlorhydria and a serum vitamin B_{12} level of 85 $\mu\mu$g./ml. the bone marrow was only equivocally megaloblastic. The pleural effusion was due to congestive cardiac failure. There were abundant mesothelial cells, macrophages, and lymphocytes, and none of these showed any morphological abnormality.

Discussion and Conclusions

We hoped by this investigation to establish whether or not there is a general visible abnormality of epithelial cells in megaloblastic anaemia, such as is implicit in the paper of Graham and Rheault (1954). It appears that, in vitamin-B_{12} deficiency and in the other anaemias which respond to folic acid, obvious abnormalities of the buccal squamous cells are the rule rather than the exception (Boddington, 1959).

Corresponding changes in the vaginal smears are difficult to establish owing to the wide variations and cyclical changes which are found normally. Our findings suggest that the nucleus can become enlarged in pernicious anaemia, but is probably only recognizable with certainty by comparing specimens before and after treatment.

The cells of the conjunctival epithelium showed no abnormalities, and those of nasal columnar epithelium only inconstant and equivocal changes. Some markedly abnormal bronchial columnar cells were seen in one sputum specimen.

The cells of the urinary tract were usually normal, but in two cases there was a reversible abnormality for which no other cause than vitamin B_{12} deficiency was found; this consisted of nuclear enlargement, a high nucleo-cytoplasmic ratio, and hyperchromasia only affecting a small proportion of the cells.

The "active" gastric epithelial cells described by Rubin were only found in one of six untreated cases, and "bland" nuclear enlargement of gastric mucosal cells was not seen in any of the gastric washings studied from this group of cases. (The features which may perhaps be specific for pernicious anaemia are the occurrence of fragments of epithelium showing intestinal metaplasia, with or without other pieces of normal mucus-secreting gastric epithelium, in the absence of pus.)

From the standpoint of diagnostic cytology it can be concluded that morphological changes of the epithelial cells in megaloblastic anaemias are less constant and less characteristic than the abnormalities found in bone marrow, and are only likely to be of diagnostic use in a very occasional case. This situation arose in Case 1, where, in spite of a macrocytic blood picture, an initial bone marrow smear was equivocal, and the abnormal cells of the saliva, urine, nasal and vaginal smears led to a further marrow puncture and serum vitamin B_{12} estimation. Boen, Molhuysen, and Steenbergen (1958) have recently described a case of subacute combined degeneration of the cord in which the examination of buccal cells gave the only morphological evidence of the disease. Cases such as these are very exceptional. The most constant changes, those of the buccal epithelium, are often indistinguishable from those found in the other types of lingual atrophy, and in the average case of pernicious anaemia the other epithelia so far examined have shown no alterations at all.

From a more fundamental standpoint, it remains to be discovered whether the nuclear enlargement represents an actual increase of nuclear solids, and whether the DNA is normal in amount. The appearances in smears do not suggest that there is an increase in DNA in the majority of cases, and the comparative ranges in nuclear size are very unlike those expected if polyploidy was the explanation. In the case of megaloblasts, Reisner (1958) has recently reviewed what evidence there is on this still unsolved problem, but it would be unjustifiable to suggest that the changes seen in the epithelial cells are of the same nature.

Summary

The cytological appearances of epithelial cells were studied in 10 cases of megaloblastic anaemia,

eight of which had typical Addisonian pernicious anaemia. Cells were examined from the mouth, nose, conjunctiva, urinary tract, and vagina both before and after treatment, and observations were also made on the gastric contents in six cases before treatment and two after. Besides the enlargement of the buccal cell nuclei which was found in eight of the cases, small numbers of abnormally enlarged cells were observed in three nasal and two urinary specimens taken before treatment. In two of three post-menopausal women there was a highly significant reduction in mean nuclear diameter of the vaginal cells after vitamin B_{12} treatment. One of the gastric washings taken before treatment showed a fragment of mucosa with irregular hyperchromatic nuclei.

All these changes were reduced or abolished by vitamin B_{12} treatment (or folic acid in the puerperal case). No abnormality was found in conjunctival cells or in macrophages.

Bronchial columnar cells with abnormally enlarged nuclei have been found in one specimen of sputum from an untreated case of pernicious anaemia.

It is concluded that widespread epithelial cell changes can occur in pernicious anaemia, but that the appearances are inconstant and non-specific.

We would like to express our thanks to Professor L. J. Witts, Dr. A. M. Cooke, Mr. J. Stallworthy, and Dr. P. D. Bedford for access to patients under their care ; also to Dr. A. H. T. Robb-Smith for reading the manuscript.

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ORAL CORNIFICATION IN ANAEMIC PATIENTS

BY

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Changes in the oral epithelium in iron-deficiency anaemia have long been recognized, and the clinical picture of glossitis and stomatitis, sometimes in association with dysphagia, is especially familiar in women with "idiopathic" hypochromic anaemia (Paterson, 1919). Waldenström (1938) has shown that these changes may occur in cases of occult iron deficiency in which anaemia has not yet developed. Despite wide appreciation of the oral symptoms little investigation of the epithelium itself in this condition has been carried out.

The normal buccal epithelium has been previously investigated by means of smears fixed and stained by Papanicolaou's technique (Jacobs, 1958). The present investigation is concerned with the buccal cornification in patients with anaemia due to iron deficiency and other causes.

A Survey of Anaemic Patients

Three groups were examined.

(1) Thirty-nine cases (18 male, 21 female) of iron-deficiency anaemia due to gastro-intestinal haemorrhage caused by haemorrhage from duodenal ulcer 13, carcinoma of large intestine nine, post-gastrectomy bleeding six, hiatus hernia five, gastric ulcer three, gastric carcinoma three.

(2) Twenty cases of "idiopathic" iron-deficiency anaemia in women all of which presented with symptoms of anaemia without any evidence of haemorrhage. In some cases there was a complaint of menorrhagia.

(3) Twenty-five cases (12 male, 13 female) of anaemia without iron deficiency, caused by pernicious anaemia seven, chronic lymphatic leukaemia six, chronic myeloid leukaemia four, myelomatosis six, and acute leukaemia two. The degree of anaemia in these cases is shown in the Table.

Papanicolaou-stained smears from the inside of the cheek were examined and the percentage of squamous cells showing cytoplasmic acidophilia or orangeophilia (cornification index) was calculated in each case.

Results

The cornification indices in the three anaemic groups are shown in Fig. 1 where they are compared with the findings in 200 normal subjects (Jacobs, 1959). The mean cornification indices of these groups are:

Normal	36.1 ± 1.4
Group 1 (post-haemorrhagic)	33.3 ± 2.7
Group 2 ("idiopathic") ..	71.9 ± 3.6
Group 3 (leukaemias and pernicious anaemia) ..	38.4 ± 3.8

The anaemic patients in Groups 1 and 3 do not vary significantly from the normal, but the increase in the cornification index in Group 2 is highly significant ($P<0.0001$). In none of the groups is there any correlation between cornification and the haemoglobin level.

The Response of Cornification to Iron Therapy

It was possible to keep four of the patients in Group 2 under prolonged observation while they

TABLE

DEGREE OF ANAEMIA IN THREE GROUPS OF ANAEMIC PATIENTS UNDER INVESTIGATION

No. of Cases	Haemoglobin (g.%)										Total
	4·0–4·9	5·0–5·9	6·0–6·9	7·0–7·9	8·0–8·9	9·0–9·9	10·0–10·9	11·0–11·9	12·0–12·9	13·0–13·9	
Group 1 ..	1	7	2	6	7	7	3	5	1	—	39
,, 2 ..	—	—	4	2	2	3	6	1	1	1	20
,, 3 ..	1	1	3	4	4	9	2	1	—	—	25

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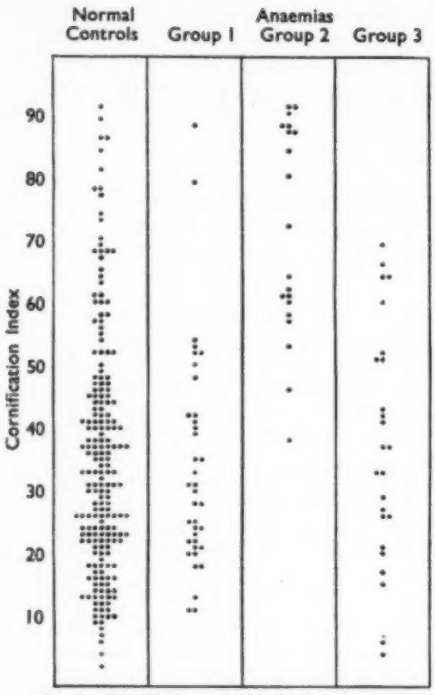

FIG. 1.—Cornification indices of 200 normal subjects compared with three groups of anaemic patients.

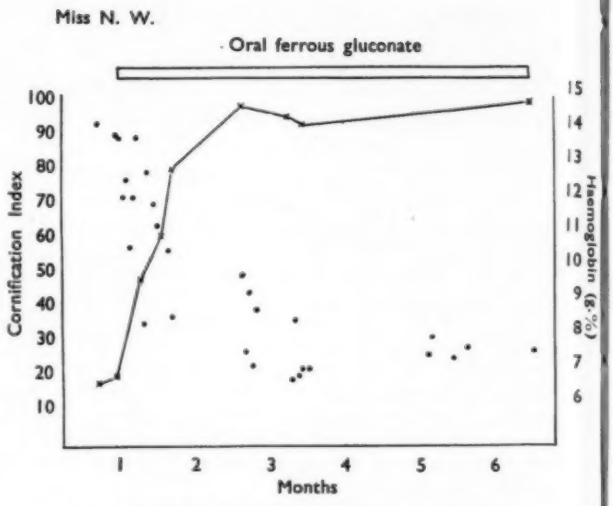

FIG. 2.—Response to oral iron therapy in Miss N. W. aged 31 years. X——X Haemoglobin. ● Cornification index.

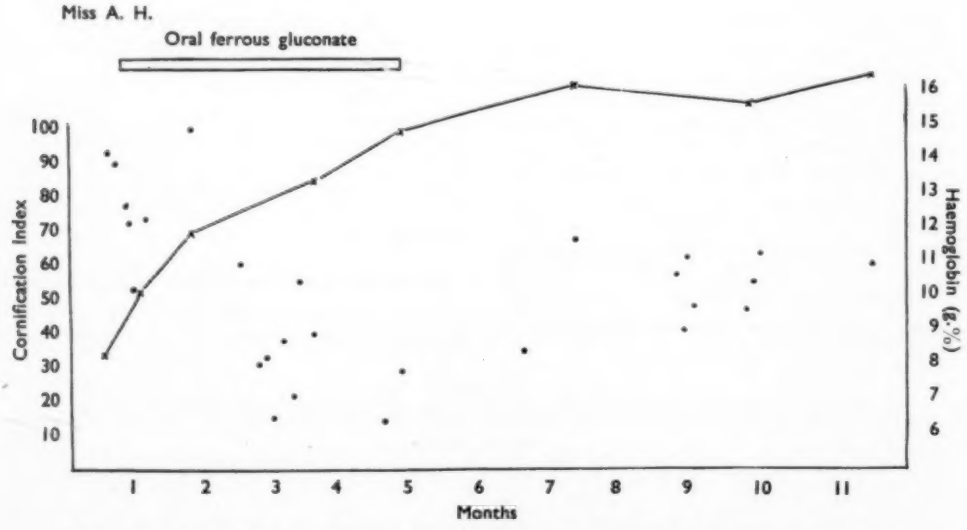

FIG. 3.—Response to oral iron therapy in Miss A. H. aged 19 years. X——X Haemoglobin. ● Cornification index.

were treated with iron. In two cases treated with ferrous gluconate, 0.6 g. t.d.s. by mouth, haematological recovery was complete and at the same time the buccal cornification fell to within normal limits (Figs. 2 and 3). Miss A. H. discontinued treatment after four months and although her blood picture remains normal there seems to be a tendency towards increased buccal cornification again. One case treated with "imferon" and another treated with both "imferon" and ferrous gluconate showed similar changes in the buccal epithelium during treatment.

Discussion

There is no evidence that buccal cornification is affected by anaemia itself. The findings in patients with leukaemia and pernicious anaemia were not different from the normal although the degree of anaemia in these cases was as severe as in the iron-deficiency groups. In the patients with iron deficiency there is no correlation between increased cornification and the severity of the anaemia.

The two groups of patients with iron-deficiency anaemia form clinically distinct entities and it is possible that the abnormal buccal cornification in the second group is related to the different development of the disease. In the first group the anaemia is known to be due to blood loss alone, but the women with "idiopathic" iron-deficiency anaemia have no evidence of haemorrhage. Menorrhagia, although sometimes associated with this syndrome, is often not severe and may be corrected by treatment of the anaemia. Other factors, including dietary defects, constitutional gastric atrophy, malabsorption of iron, and excessive childbearing, have been invoked to explain the pathogenesis of this disorder. The essential feature of this "idiopathic" anaemia, whatever its primary cause, is its chronicity (Moore, 1950).

The epithelial changes noted above may be related to the tissue stores of iron rather than to the anaemia itself. In pernicious anaemia and the leukaemias there is no excess loss of iron from the body and the reserves may even be increased if blood transfusions have been given. In "idiopathic" hypochromic anaemia the disease usually pursues a chronic relapsing course—often over several decades. Patients with this disorder tolerate low haemoglobin levels for long periods before seeking medical treatment and then do so only because the severity of the anaemia makes normal life impossible. These patients have grossly depleted iron stores which probably cannot be fully replenished by oral iron therapy alone. Cases of gastro-intestinal haemorrhage do not commonly remain untreated long enough for the iron stores to become so grossly depleted. Where such cases do remain undetected for a long period the patient will probably present as a case of "idiopathic" anaemia with the associated oral changes.

Exhaustion of the body's supply of iron will lead to an intracellular deficiency and consequent disorder of cellular metabolism. Epithelial cornification seems to be affected by this disorder and the return of buccal cornification to normal under the influence of iron therapy indicates that iron is in some way concerned with the normal development of squamous epithelium.

Some cases showing a high cornification index were found in the healthy controls, but as they were not investigated haematologically it is quite possible that some subjects with overt or latent iron deficiency were included in this group.

Summary

The buccal cornification, as reflected in Papanicolaou-stained smears, has been estimated in patients with iron-deficiency and other anaemias.

Women with "idiopathic" hypochromic anaemia show an abnormal increase in buccal cornification which has been shown in some cases to revert to normal after iron therapy. This is not related to the degree of anaemia but may be due to decreased tissue iron stores.

I wish to thank Dr. S. Shaw for his encouragement in this work.

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OBSERVATIONS ON THE AMINO-ACIDURIA IN MEGALOBLASTIC ANAEMIA

BY

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An abnormal excretion of amino-acids in the urine of patients with Addisonian pernicious anaemia in relapse has been reported by Weaver and Neill (1954), by Keeley and Politzer (1956), and by Crane, Hayes, and de Gruchy (1958). Weaver and Neill (1954) stated that in five patients there was an abnormal excretion of taurine with some over-excretion of lysine, cystine, and leucine, while in another with subacute combined degeneration of the spinal cord with no anaemia there was an abnormal excretion of taurine. Keeley and Politzer (1956) confirmed these findings in two patients with Addisonian pernicious anaemia and reported that in seven African patients with non-Addisonian megaloblastic anaemia the urinary amino-acid patterns were normal. On the other hand, Crane *et al.* (1958), after investigating nine patients with untreated Addisonian pernicious anaemia of whom two had subacute combined degeneration of the spinal cord, concluded that although amino-aciduria did occur there was no characteristic urinary amino-acid pattern in these patients. Moreover, one patient with subacute combined degeneration of the spinal cord showed no abnormal amino-acid excretion. They reported an increased excretion of taurine and β-amino-iso-butyric acid (BAIB) in about half of their patients, and abnormal traces of aspartic acid, leucine, lysine, phenylalanine, tyrosine, and valine were also present. All three groups of investigators employed paper chromatographic techniques and studied urines only. The present report describes the results of an investigation of the urinary and plasma amino-acid patterns in a comparatively large series of patients with Addisonian pernicious anaemia and other megaloblastic anaemias. An attempt was made to determine the incidence and extent of the amino-aciduria; the consistency of the abnormal excretion of taurine; the nature of the amino-aciduria; and the diagnostic value, if any, of such studies.

*This investigation was carried out during the tenure of a Sino-British Fellowship Trust Scholarship.

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MATERIAL AND METHODS

The patients investigated were of two groups.

Megaloblastic Anaemia Due to Vitamin B_{12} Deficiency

In this group were 22 patients with classical Addisonian pernicious anaemia in whom the diagnosis was made on the finding of a megaloblastic bone marrow, histamine-fast achlorhydria, a low serum vitamin B_{12} level, and a haematological response to vitamin B_{12}. Also included were two patients suffering from the malabsorption syndrome with megaloblastic bone marrow changes, low serum vitamin B_{12} levels, free acid in the gastric juice, and satisfactory haematological responses to vitamin B_{12}. All patients were investigated before and after the return of the blood levels to normal after treatment.

Megaloblastic Anaemia of Puerperium/Pregnancy

There were nine patients in whom only one was first seen before delivery. The diagnosis was based on the findings of a megaloblastic bone marrow, free acid in the gastric juice, a normal serum vitamin B_{12} level, and a satisfactory haematological response to folic acid alone. Seven of these patients were investigated both before treatment and after treatment when the blood levels had returned to normal.

Controls

Non-anaemic Patients. — Eight haematologically normal patients served as non-anaemic controls. No acutely ill or febrile patient was included.

Anaemic Patients.—This group included:

(i) Five patients with iron-deficiency anaemia from chronic blood loss (three from duodenal ulcer and two from menorrhagia) of whom the two most severely anaemic (haemoglobin=5.62, 5.93 g./100 ml.) were investigated both before and after treatment.

(ii) Two patients with idiopathic hypoplastic anaemia (haemoglobin 5.92 and 6.82 g./100 ml.).

(iii) Two patients with acute idiopathic acquired haemolytic anaemia (Coombs test positive in both).

(iv) One patient with anaemia due to chronic myeloid leukaemia (haemoglobin 9.47/100 ml., W.B.C. 340,000/c.mm., and platelets 322,000/c.mm.).

All patients were on the usual hospital diet. Twenty-four hour urine collections were preserved with thymol crystals and refrigerated. Collections were made before the beginning of specific therapy and at different intervals afterwards. A final collection was made when there had been a satisfactory haematological response to therapy. Plasma from heparinized venous blood was obtained both before and after treatment. Heparinized plasma was also separated from bone marrow blood in the course of the diagnostic sternal puncture and a venous blood specimen was obtained at the same time for comparative studies.

The total α-amino nitrogen in the 24-hour urine specimens was determined by the formal titration method of Van Slyke and Kirk (1933) taking special precaution to distil off *in vacuo* all ammonia as tested by red litmus paper.

The individual amino-acids were identified by ascending 2-dimensional paper chromatography employing essentially the technique described by Dent (1951). Whatman No. 1 or No. 4 paper was cut to the size of 30×30 cm. and the two solvents used were water-saturated phenol and lutidine-water. The "2-second" urine (the actual volumes varied from 15 to 50 μl.) and 625 μl. of deproteinized (by the addition of 10 volumes of 95% ethyl alcohol) and electrolytically desalted plasma were the amounts applied for chromatography. Preliminary oxidation with ammonium molybdate and hydrogen peroxide was carried out for the detection of methionine and cystine. The chromatograms were sprayed with an 0.1% solution of ninhydrin in butanol and developed at 100° C. for 10 minutes. Heating was found to yield a more complete pattern of ninhydrin-positive spots than developing at room temperature. A rough quantitative estimation of the amino-acids was made by comparison with spots given by 5, 10, 20, 40 μg. of taurine. One-dimensional runs in butanol-acetic acid-water were examined for the presence of histidine and tyrosine after spraying with Pauly's diazo reagent.

Liver function tests performed included the estimation of serum albumin, globulin, bilirubin, and alkaline phosphatase levels, and the thymol turbidity and colloidal gold flocculation tests.

RESULTS

Total α-Amino Nitrogen in Urine

The mean total α-amino nitrogen concentrations in 24-hour urine collections of the patients so investigated are set out in Table I. The levels in the three groups of anaemic patients before treatment were not significantly different from that of the controls. However, the mean level in

TABLE I

TOTAL URINARY α-AMINO NITROGEN EXCRETION IN 24 HOURS IN DIFFERENT PATIENTS

	No. of Patients	α-Amino Nitrogen (mg./24 Hours)
Controls	7	251·6±35·5
Pernicious anaemia:		
(a) Before treatment	9	214·7±39·1
(b) After ,,	9	164·8±5·9
Iron-deficiency anaemia	3	290·7±58·8
Megaloblastic anaemia of puerperium	3	211·3±52·8

TABLE II

CONCENTRATIONS OF URINARY AMINO-ACIDS IN EXCESS IN DIFFERENT PATIENTS BEFORE TREATMENT

Patient	Sex and Age	Initial Haemoglobin (g./100 ml.)	Amino-acid in Excess (μg.)
Addisonian Pernicious Anaemia			
E. A.	F 28	4·15	Taurine 10; cysteic acid 5; histidine 5; trace amounts of glutamic acid, glutamine, BAIB, arginine, lysine, leucines, valine, and α-aminobutyric acid
S. M.	F 65	5·77	Taurine 5
A. W.	F 61	5·92	,, 5; glycine 5
J. W.	M 63	6·50	,, 5; BAIB trace
H. L. T.	M 52	6·50	,, 5; glycine 5
O. P.	M 52	6·50	,, 5
J. J.	F 72	6·66	,, trace
J. A.	F 60	6·82	,, 5
A. D.	F 72	6·96	,, 5
D. R.	M 72	6·96	,, 10; histidine 5
A. D.	M 72	6·96	,, 5; BAIB 5
G. L.	M 61	7·55	,, 5; glycine 5
R. C.	M 67	7·70	None in excess
J. M.	M 54	8·74	Taurine 10
S. S.	F 50	8·74	,, trace; serine trace
M. W. (Subacute combined degeneration of the spinal cord)	F 47	9·66	,, 5; glycine 5; traces of serine and BAIB
A. W.	M 74	9·66	Taurine trace
M. H.	F 65	9·77	,, 5; alanine trace
L. B.	M 68	9·77	,, 5; glycine 5; alanine 5; glutamic acid trace
E. M.	F 67	10·05	Taurine 5; glycine 5; BAIB 5; glutamine trace
S. M.	F 49	11·40	Taurine 5; glutamine trace
M. K.	F 57	11·84	,, trace
Malabsorption Syndrome and Megaloblastic Anaemia			
E. M.	F 58	9·92	Taurine 5; BAIB trace
R. E.	M 79	11·84	None in excess
Megaloblastic Anaemia of Pregnancy/Puerperium			
M. T.	F 29	5·63	Taurine trace; BAIB trace
M. C. (Pregnant)	F 30	5·77	Glycine 5; threonine 5; BAIB 5; traces of alanine and α-aminobutyric acid
M. G.	F 21	6·67	None in excess
I. M.	F 29	6·82	BAIB trace
M. M.	F 32	7·10	Traces of taurine and leucines
A. C.	F 32	7·85	Traces of taurine, threonine, BAIB, valine and cysteic acid
M. D.	F 20	10·65	None in excess
Iron-deficiency Anaemia			
M. G.	F 25	5·93	Taurine 5
A. P.	F 25	5·62	,, 5; BAIB trace
Acquired Haemolytic Anaemia			
M. L.	F 63	4·44	Taurine trace; traces of threonine, leucines, phenylalanine, and valine
J. H.	F 61	6·22	Taurine 5; BAIB 5; traces of lysine, serine, and leucine

the patients with pernicious anaemia after treatment was significantly lower than that encountered in the same patients before treatment and in the controls.

Paper Chromatographic Studies on Urine in Patients

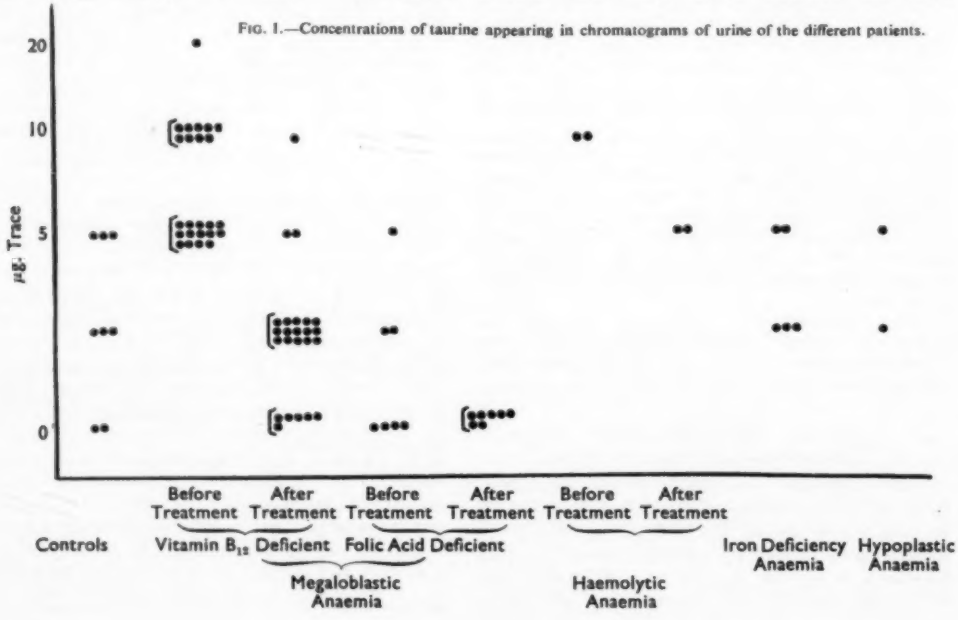
Megaloblastic Anaemia due to Vitamin B_{12} Deficiency. — The patients with classical Addisonian pernicious anaemia and those with megaloblastic anaemia from vitamin B_{12} deficiency associated with the malabsorption syndrome are considered as one group because the results they showed were essentially the same (Table II). One patient had the features of subacute combined degeneration of the spinal cord.

The most consistent abnormality in these patients before treatment was an over-excretion of taurine which disappeared after vitamin B_{12} therapy (Fig. 1). This was observed in 22 of the 24 patients. In the other two taurine corresponding to 5 μg. was present initially but did not show any diminution after treatment; in one the initial haemoglobin level was 11.84 g./100 ml. In four of these patients the 24-hour urines were examined at weekly intervals for four weeks. It was found that by the third week after the start of vitamin B_{12} therapy the urinary chromatogram had assumed the normal pattern and that the decrease in taurine was a consistent feature.

A significant over-excretion of BAIB, glycine, glutamine, glutamic acid, histidine, serine, and alanine singly or in various combinations was present in seven of the 24 patients (Table II). However, a generalized amino-aciduria occurred in one only (E. A., F., 28) and this patient had the lowest haemoglobin level (4.15 g./100 ml.) in the present series. An excess of BAIB was present in only six of the 24 patients, although marked anorexia was admitted by 22 of the 24 patients. This is at variance with the suggestion of Crane *et al.* (1958) that increased BAIB excretion in these patients may be the result of anorexia and general malaise. The increased amounts of glycine and histidine sometimes observed in the chromatograms in some patients before treatment may be attributed to variations in diet and are probably of no real significance.

There was no correlation between the degree of anaemia and the amino-aciduria with the exception that the most profoundly anaemic patient showed the most generalized amino-acid over-excretion. There was no correlation between the level of serum vitamin B_{12} and the extent of the amino-aciduria. Liver function tests performed in seven of these 10 patients were normal and these seven included the most

FIG. 1.—Concentrations of taurine appearing in chromatograms of urine of the different patients.



profoundly anaemic patient (E.A., F., 28). There was a slight rise in globulin in the other three patients and of these one had a positive thymol turbidity test. However, they (A. D., F., 72; D. R., M., 72; and A. D., M., 72) did not show urinary amino-acid excretion patterns significantly different from those of the others.

Megaloblastic Anaemia of Puerperium/Pregnancy.—Of the nine patients investigated, three showed no abnormality in the urinary amino-acid patterns. Only seven of these nine patients were investigated both before and after treatment (Table II). One of the patients (M. C., F., 30) was pregnant when first seen and the amino-aciduria detected may have been wholly or in part attributable to the pregnancy (Wallraff, Brodie, and Borden, 1950). Of the remaining six patients, one had abnormal features in the liver function tests (M. T., F., 29: thymol turbidity = 9 Maclagan units, serum albumin low), but it is unlikely that this was responsible for the slight increase in excretion of taurine and BAIB, for these disappeared after treatment with folic acid while the liver function tests remained abnormal. Furthermore, the liver function tests were normal in two of the other patients (I. M., F., 29, and M. M., F., 32). It is not known whether or not amino-aciduria is present in the puerperium which might account for the slight increases in BAIB, taurine, cystine, leucine, and/or valine encountered. An interesting feature was that taurine was not detected in six of the nine patients before treatment and that when present it was never as great in amount as that observed in the patients with pernicious anaemia (Fig. 1). After treatment taurine was no longer detected in any of the chromatograms.

As in the patients with pernicious anaemia there was no apparent correlation between the severity of the anaemia and the appearance of amino-aciduria, but the relatively small number of patients and similarities in their haemoglobin levels do not permit of a definite conclusion.

Paper Chromatographic Studies in Controls

Non-anaemic Patients.—The chromatograms of the urine from these patients were similar to those described as occurring in the normal by Walshe (1953) and Dent (1954), and showed glycine as the predominant spot with smaller amounts of histidine, alanine (in six), taurine (in six), glutamic acid (in five), cysteic acid, traces only (in five), methyl histidine (in three), BAIB (in two), and glutamine (in one). The intensity of the taurine spot in no instance exceeded that corresponding to 5 μg. of pure taurine.

Anaemic Patients.—

(i) *Iron-deficiency Anaemia.*—There were no abnormal features in the urinary amino-acid patterns in three patients with haemoglobin levels above 9 g./100 ml. On the other hand, two patients with haemoglobin levels below 6 g./100 ml. showed a slightly increased excretion of taurine and this was associated with a slight increase in BAIB in one (Table II). These diminished with adequate iron therapy. Both had normal serum vitamin B_{12} levels.

(ii) *Aplastic Anaemia.*—In the two patients investigated there was no abnormality in the urinary amino-acid patterns. In one taurine was detected in normal amounts.

(iii) *Acquired Haemolytic Anaemia.*—The two patients investigated both showed a mild generalized amino-aciduria (Table II). In both the amino-aciduria diminished as the haemoglobin increased following prednisone therapy. In one the urinary amino-acid pattern became normal, but in the other, in whom the haemolytic process was less satisfactorily controlled, the amino-aciduria diminished but did not completely disappear. In this latter patient the bone marrow was initially megaloblastic with a normal serum vitamin B_{12} level of 120 $\mu\mu$g./ml. Liver function tests were performed in one of these patients (J. H., F., 61), and the results were normal apart from a bilirubinaemia.

(iv) *Chronic Myeloid Leukaemia.* — The chromatogram of this patient's urine showed a normal amino-acid pattern and no taurine.

Chromatographic Studies on Venous Blood Plasma

The plasma from eight patients with pernicious anaemia examined by paper chromatography both before and after treatment showed no significant difference in the amino-acid patterns. Only trace amounts of taurine were detected, and in no instance was it greater than 5 μg. Furthermore, these plasma chromatograms did not differ significantly from those of four normal controls, two patients with megaloblastic anaemia of puerperium, two patients with iron deficiency anaemia, and two patients with acquired haemolytic anaemia.

On the other hand, the plasma chromatogram of the patient with chronic myeloid leukaemia revealed an excess of taurine and glutamic acid (Table III). This latter finding is in accordance with the report by Kelley and Waisman (1957) that in chronic myeloid leukaemia the plasma glutamic acid may be increased. It should be

noted, however, that the chromatogram of this patient's urine did not reveal the presence of taurine (*v. supra*).

Chromatographic Studies on Bone Marrow Blood Plasma

The plasma from bone marrow blood of seven patients was examined chromatographically and compared with the chromatograms of plasma from peripheral venous blood withdrawn at the same time. The results show that with the exception of taurine and glutamic acid the amino-acid patterns were the same. These two amino-acids gave much larger spots in the chromatograms of the marrow plasma than in those of the venous plasma (Table III). This increase in taurine and glutamic acid was the same in the patients with pernicious anaemia, iron-deficiency anaemia, and acquired haemolytic anaemia, but was more marked in the marrow plasma of the patient with chronic myeloid leukaemia.

TABLE III

CONCENTRATIONS OF TAURINE AND GLUTAMIC ACID IN BONE MARROW PLASMA AND VENOUS BLOOD PLASMA IN DIFFERENT PATIENTS

	Taurine (μg.)		Glutamic Acid (μg.)	
	Marrow Plasma	Venous Plasma	Marrow Plasma	Venous Plasma
Pernicious anaemia:				
A. D.	5	Trace	20	5
M. M.	5	,,	20	Trace
Iron-deficiency anaemia:				
M. G.	10	,,	10	,,
P. N.	10	,,	20	,,
Acquired haemolytic anaemia:				
J. H.	10	,,	20	,,
M. L.	5	,,	10	,,
Chronic myeloid leukaemia:				
M. G.	>40	30	>40	>40

The significance of these results is uncertain. While it is admitted that there was no means by which one could aspirate amounts of bone marrow comparable in cellular and plasma content each time, the consistency of the finding of an increase in taurine and in glutamic acid is of interest. However, it is felt that this may be attributable to the larger amounts of leucocytes and platelets in the marrow as compared with peripheral blood, and to the slight haemolysis that occurred in the course of handling of the marrow specimens. This is suggested by the report of McMenamy, Lund, and Oncley (1957) that leucocytes and platelets contain a large amount of taurine and that glutamic acid may leak from erythrocytes during the handling of whole blood. The findings in the marrow plasma from the patient with chronic myeloid leukaemia (Table III) in which there were greatly increased numbers of leucocytes and platelets further support this contention.

DISCUSSION

The results of the total urinary α-amino-nitrogen estimations show that this is not significantly increased in patients with megaloblastic anaemia from either vitamin B_{12} or folic acid deficiency. Therefore the chromatographic results may be interpreted as showing qualitative rather than quantitative changes in the urinary amino-acids. However, this does not indicate that the excretion of individual amino-acids may not be in excess of normal, and although paper chromatographic studies are at best only semi-quantitative the results of the present investigation suggest that this does occur. The significantly lower excretion of total α-amino nitrogen in the urine of the patients with pernicious anaemia after treatment may be the result of the nitrogen retention which occurs in these patients following effective therapy (Baldbridge and Barer, 1931).

Analysis of the results suggests that in the patients with vitamin B_{12} deficiency there are two abnormalities in the urinary amino-acid excretion pattern. The most consistent of these, occurring in 22 of the 24 patients investigated, was an over-excretion of taurine and this is in accord with reports by Weaver and Neill (1954), Keeley and Politzer (1956), and Crane *et al.* (1958). This over-excretion of taurine occurred alone or in association with an over-excretion of other amino-acids. It was not encountered in other patients with comparable degrees of anaemia, with the exception of patients with acute idiopathic haemolytic anaemia where it occurred together with a generalized amino-aciduria. The second abnormality, encountered in only seven of the 24 patients, consisted of a slight but significant over-excretion of BAIB, glycine, histidine, glutamine, alanine, glutamic acid, and serine in varying combinations amounting to a generalized amino-aciduria in the most severely anaemic patient. This abnormality was also present in the patients with megaloblastic anaemia of puerperium/pregnancy and acquired haemolytic anaemia, and to a lesser extent in a patient with severe iron-deficiency anaemia (Table II). It was not encountered in the two patients with hypoplastic anaemia.

The cause of the over-excretion of taurine in these patients with vitamin B_{12} deficiency is uncertain. The presence of a larger amount of

taurine in marrow plasma when compared with venous plasma has been discussed and attributed to contamination by the larger number of leucocytes and platelets in the former. It is possible that it was due to hepatic dysfunction. The normal plasma taurine levels do not exclude this, for, according to Dent (1958), plasma levels of taurine are normally low and difficult to detect chromatographically, and as renal taurine clearance is very high it is possible to have an overflow taurinuria with very little detectable in the blood plasma. However, there was no correlation between abnormal liver function tests and the increase in urinary taurine, and furthermore there was no excess excretion of cystine described as occurring in hepatic disease (Walshe, 1953). Another possible mechanism in the increase in urinary taurine is that it was due to a renal tubular defect. This seems unlikely as it would have then occurred as an isolated urinary taurinuria often marked and yet unassociated with over-excretion of other amino-acids. According to Dent (1958) taurine may be excreted in increased amounts in conditions associated with a negative nitrogen balance which has been reported to occur in patients with pernicious anaemia (Alt, 1929). This may account in part for the present findings and may also explain the increased taurine excretion in the patients with severe iron-deficiency anaemia. However, that this is probably not the entire explanation is shown by the absence of a comparable increase in taurine excretion in the patients with megaloblastic anaemia of the puerperium, when a negative nitrogen balance is often present (Cantarow and Trumper, 1949). Crane *et al.* (1958) suggested, on the basis of the report by Jukes, Stokstad, and Broquist (1950) that in chicks vitamin B_{12} may be required for the transformation of homocysteine to methionine, that a lowered rate of synthesis of methionine due to vitamin B_{12} deficiency may result in an enhanced urinary excretion of taurine through degradation of accumulated homocysteine in the body (Eldjarn, 1954). It is at present considered that this, together with a negative nitrogen balance, may be the explanation for the increased urinary taurine observed in these patients with vitamin B_{12} deficiency.

The increased taurine excretion in the patients with acute acquired haemolytic anaemia was not related to vitamin B_{12} deficiency, for in both the serum vitamin B_{12} levels were over 100 $\mu\mu$g./ml. In all probability it occurred as part of a generalized amino-aciduria.

An over-excretion of one or more of the other amino-acids was present in only seven of the 24 patients with vitamin B_{12} deficiency, but this was not characteristic of this group as it also occurred in patients with a megaloblastic anaemia of the puerperium/pregnancy, acquired haemolytic anaemia, and, to a lesser degree, in severe iron-deficiency anaemia. The arguments against this being the result of bone marrow or hepatic dysfunction are the same as those discussed in connexion with taurine. In the patient first seen in pregnancy the amino-aciduria may have been due to the pregnancy (Wallraff *et al.*, 1950). In the puerperal patient the mild amino-aciduria was probably not due to the puerperium because in two patients (M. G., F., 21 ; M. D., F., 20) both first seen within three weeks of delivery there was no abnormality in the urinary chromatograms (Table II). Anaemia alone does not appear to be the explanation, for there was no amino-aciduria in the patients with aplastic anaemia and comparable haemoglobin levels.

In view of the normal plasma concentration of the amino-acids detected in excess in the urine of these patients, it seems reasonable to suggest that the amino-aciduria encountered was of renal origin. Renal tubular dysfunction has been described in severe Addisonian pernicious anaemia by Stieglitz (1924) and in acute haemolytic anaemia by Dacie (1954), and that renal tubular dysfunction may result in amino-aciduria of varying degrees can be concluded from the reports by Spencer and Franglen (1952), Wilson, Thompson, and Dent (1953), Pare and Sandler (1954), and Marsden and Wilson (1955). In this connexion the report of renal amino-aciduria in ascorbic acid deficiency by Jonxis and Huisman (1954) is of interest, for ascorbic acid is intimately related to amino-acid metabolism (Bicknell and Prescott, 1953). The presence of amino-aciduria in the less severely anaemic patients is not so readily explained by renal tubular dysfunction, but it may well be that the primary defect is a disorder in amino-acid metabolism at a cellular level involving body cells in general and that the demonstration of amino-aciduria is merely due to the accessibility of the kidneys by means of urinary examination. It has been shown that there is a close relation between amino-acid metabolism and erythropoiesis, although the precise role of each amino-acid is yet to be determined (Wintrobe, 1956). Therefore, while the underlying mechanism for the amino-aciduria in these patients with abnormal erythropoiesis remains uncertain, it is suggested that it is a

manifestation of a general disturbance in amino-acid utilization and degradation in the cells of the body as a whole.

The reports of Weaver and Neill (1954) and Keeley and Politzer (1956) suggested that paper chromatography of urine may afford a means by which megaloblastic anaemia from vitamin B_{12} deficiency could be differentiated from that due to folic acid deficiency. The present findings indicate that the demonstration of a mild amino-aciduria is specific neither for vitamin-B_{12}-deficient nor folic-acid-deficient patients. It may or may not be present in either. The results would be even more difficult to interpret in pregnancy when a megaloblastic anaemia sometimes develops and the question of choice of treatment with either vitamin B_{12} or folic acid arises. As to the increase in taurine excretion, reference to Fig. 1 shows that this occurs regularly in patients with B_{12} deficiency, and not in those deficient in folic acid. Therefore the demonstration of a large taurine spot of over 5 μg. intensity would favour the diagnosis of vitamin B_{12} deficiency in a patient with megaloblastic anaemia. However, prediction in the individual case may be open to fallacies as there is some overlapping amongst those with smaller degrees of taurinuria (Fig. 1). Moreover, taurine over-excretion may occur in a variety of conditions such as fever, cancer, and wasting diseases (Dent, 1958) and in acute acquired haemolytic anaemia. It is, therefore, concluded that when marked taurine excretion is encountered in a patient with megaloblastic anaemia the diagnosis of primary vitamin B_{12} deficiency rather than folic acid deficiency may be made provided all other causes of increased urinary taurine excretion can be ruled out.

Lastly, the urinary amino-acid pattern of the only patient in this series with subacute combined degeneration of the spinal cord was not different from that encountered in patients with Addisonian pernicious anaemia without neurological complications. Unfortunately no patient suffering from subacute combined degeneration of the spinal cord without anaemia was investigated.

SUMMARY AND CONCLUSIONS

The 24-hour excretion of total α-amino nitrogen was normal in patients with megaloblastic anaemia due to vitamin B_{12} deficiency (Addisonian pernicious anaemia and the malabsorption syndrome) and folic acid deficiency (anaemia of pregnancy and the puerperium) before treatment.

The mild amino-aciduria encountered before treatment in megaloblastic anaemia due to vitamin B_{12} deficiency was non-specific and occurred to a similar degree in megaloblastic anaemia due to folic acid deficiency and in acute acquired idiopathic haemolytic anaemia.

An increase in urinary taurine occurred more regularly and was more pronounced in megaloblastic anaemia due to vitamin B_{12} deficiency before treatment, and, if interpreted with caution, may be helpful in differentiating it from megaloblastic anaemia due to folic acid deficiency.

The mild amino-aciduria and increase in urinary taurine decreased after specific therapy.

The possible causes of the amino-aciduria are discussed.

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AMINO-ACID TOLERANCE CURVES AND AMINO-ACIDURIA IN COOLEY'S AND SICKLE-CELL ANAEMIAS

BY

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This study was prompted by the previous finding of amino-aciduria (Choremis, Zannos, and Basti, 1957) and qualitative and quantitative changes of serum proteins in thalassaemia and sickle-cell anaemia (Allamanis, 1955; Afentaki, 1949; Minnich, Na-Nakorn, Chongchareonsuk, and Kochaseni, 1954). The electrophoretic pattern shows a fall in albumin, a moderate fall in the α_1 α_2, and β globulin fractions and an increase in the γ globulin fraction (Allamanis, 1955). In some cases liver function tests (thymol turbidity and cephalin-cholesterol flocculation tests) are positive (Choremis and Kyriakidou, 1954; Minnich *et al.*, 1954). This abnormal electrophoretic pattern, and especially the increase in γ globulin, does not necessarily indicate liver damage, although this may occur.

In order to elucidate further the amino-acid metabolism in Cooley's and sickle-cell anaemia total plasma amino-acids and urine paper chromatography studies for amino-acids were undertaken after oral administration of protein and protein hydrolysate, as well as after intravenous injection of amino-acids. In this way the proteolytic activity of the gastro-intestinal tract, the absorption of amino-acids, their intermediate metabolism and their rate of disappearance from the blood stream, and especially their excretion through the kidneys, were studied.

There are no references in the available literature to any study on the metabolism of amino-acids in thalassaemia and sickle-cell anaemia.

In a previous paper, the above observations stimulated an investigation of another aspect of protein metabolism in these anaemias, namely, the amino-acids in blood serum and their excretion in the urine (Choremis *et al.*, 1957). A few reports have appeared, however, dealing with amino-aciduria in other types of anaemia. Souchon and Grunau (1952) described one case of congenital spherocytic anaemia and cholelithiasis with amino-aciduria which was considered to be due both to liver damage and to faulty reabsorption by the tubules. Weaver and Neill (1954) reported five cases of pernicious anaemia with abnormal excretion of taurine and some overexcretion of lysine, leucine, and cystine. This type of amino-aciduria was attributed to lack of vitamin B_{12} which is known to be involved in methyl synthesis and transmethylation of certain amino-acids. The same authors also reported five cases of other varieties of anaemia without any pathological findings. Pare and Sandler (1954) described 12 cases of March haemoglobinuria with amino-aciduria. In the above cases the renal tubular defect was considered to be the cause of the abnormal amino-aciduria (overexcretion of cystine in all cases and of β-amino-isobutyric acid in 11 of the 12 cases).

Material

Ten patients with Cooley's anaemia, aged 6 months to 4 years, with haemoglobin levels between 3.4 and 9.3 g.% and red blood cell counts of 1,260,000 to 3,100,000 per c.mm. were examined. Blood urea was normal in all cases, ranging from 16 to 32 mg./100 ml. Thymol turbidity and cephalin-cholesterol flocculation tests were negative in eight of the 10 cases. Seven of the patients had recently had blood transfusions. Two had undergone splenectomy more than two years previously.

The second group was of 10 cases of sickle-cell anaemia, aged 3 to 12 years, with haemoglobin levels between 3 and 8.6 g.% and red cell counts of 860,000 to 2,500,000 per c.mm. The blood urea level was normal in all cases, ranging from 19 to 35 mg. per 100 ml. Thymol turbidity and cephalin-cholesterol flocculation tests were negative in most cases. Five of the patients had recently had blood transfusions. Three had undergone splenectomy more than one year before.

Ten normal children, aged 6 months to 8 years, were used as controls.

No glycosuria was found in any of the three groups.

Method

The plasma amino-nitrogen was determined by the chromatographic method of Frame, Russell, and Wilhelmi (1943), as modified by Fister (1948). The accuracy of the method was assessed by doing 200 determinations on four samples of known amino-N content (4, 8, 12, and 16 mg.).

For the study of urinary amino-acids paper chromatography as described by Consden, Gordon, and Martin (1944) and Dent (1947, 1948) with a slight modification was used.

Whatman No. 3 filter paper was used for the urine chromatograms and a volume of urine containing 500 μg. of nitrogen. The urine was first treated with ammonium molybdate and nitrogen peroxide on the paper, after which two-dimensional ascending chromatography was carried out employing phenol-water as the first solvent and pyridine-amyl-alcohol-water as the second. Sodium cyanide and ammonium hydroxide were added to the phenol box and diethylamine to the pyridine box. Subsequently, the papers were dried, sprayed with a 0.1% ninhydrin solution and dried at room temperature for 24 hours. The amino-acid spots thus developed were identified according to their position on the paper. Their colour intensity was compared with the test spots of pure taurine.

Although two-dimensional paper chromatography is very useful for qualitative determination, it does not otherwise provide an accurate quantitative basis for the estimation of amino-acids due to the varying sensitivity of the latter to ninhydrin. More accurate quantitative estimations of amino-acids by microbiological assays or chemical methods (Van Slyke) were not undertaken in the present study.

Plasma amino-N determination and urine paper chromatography studies for amino-acids were run for all three groups after administering (*a*) calcium caseinate ("casec") orally, (*b*) protein hydrolysate ("nesmida") orally, and (*c*) casein hydrolysate ("amigen") by intravenous injection.

The experiment was carried out as follows:

(*a*) *Oral Calcium Caseinate.*—The children were starved for 12 hours, after which the first sample of blood was taken. Then a protein meal was given by gavage, consisting of calcium caseinate, which contains 88% protein. The amount given was 1.72 g. (=1.5 g. of protein=235 mg. of nitrogen) per kilogram of body weight diluted in 30 ml. of water and 5% sugar added. Subsequent samples of blood were taken 30, 75, 150, and 240 minutes after the completion of gavage.

Urine was collected before the gavage of the test meal as well as between 0 and 75, 75 and 150, and 150 and 240 minutes after the test meal.

(*b*) *Oral Protein Hydrolysate.*—The experiment was carried out as previously described, the only difference being the use of powder protein hydrolysate, which contains 88% protein. The amount given was 2 g. (=1.5 g. of protein=230 mg. of nitrogen) per kilogram of body weight.

(*c*) *Intravenous Casein Hydrolysate.*—Amigen, 3 ml. per kg. body weight, was injected. The subsequent samples were taken from a different vein five, 15, 30, and 90 minutes after the injection had been completed.

Urine was collected before the injection as well as between 0 and 15, 15 and 30, and 30 and 90 minutes after it.

During the above period no food was given to the children except water, and any vomiting or diarrhoea, even of the slightest, cancelled the test for that day.

Children with fever or diarrhoea, or who had been given sulphonamide drugs for the last three days preceding the experiment, were excluded.

Results

The mean amino-nitrogen curves after the ingestion of calcium caseinate are shown in Fig. 1.

As can be seen in all tested children, there was an increase of the plasma amino-N levels of approximately 2 mg. per 100 ml. above the fasting level in at least one post-prandial sample. No difference has been noted in the plasma amino-N levels 75 minutes after the ingestion of calcium caseinate between the healthy controls and patients with Cooley's or sickle-cell anaemias. In most cases the highest level of the plasma amino-N was 75 minutes after the test meal. The plasma amino-N level declines gradually after 75 to 150 minutes in healthy controls, whereas in Cooley's and sickle-cell anaemias the fall is abrupt.

It was finally noted that 240 minutes after the protein meal the mean plasma amino-N value in healthy controls did not approach the original fasting level; this might be due to continuation of hydrolysis of the intact protein from some time after the four-hour test period. On the other hand, in Cooley's and sickle-cell anaemias, the mean amino-N value did approach the original fasting level 240 minutes post-prandially.

Fig. 2 is a histogram of the elimination of urinary amino-acids after the ingestion of calcium caseinate.

The greatest elimination of amino-acids is observed, as a rule, between 75 and 150 minutes after the ingestion of calcium caseinate; in normal children (13–15 spots of amino-acids, with colour intensity ranging between that given by 10 and 80 g. of taurine), and in those suffering from Cooley's or sickle-cell anaemias (13–18 spots of amino-acids, with colour intensity ranging from just above normal to considerably over the intensity

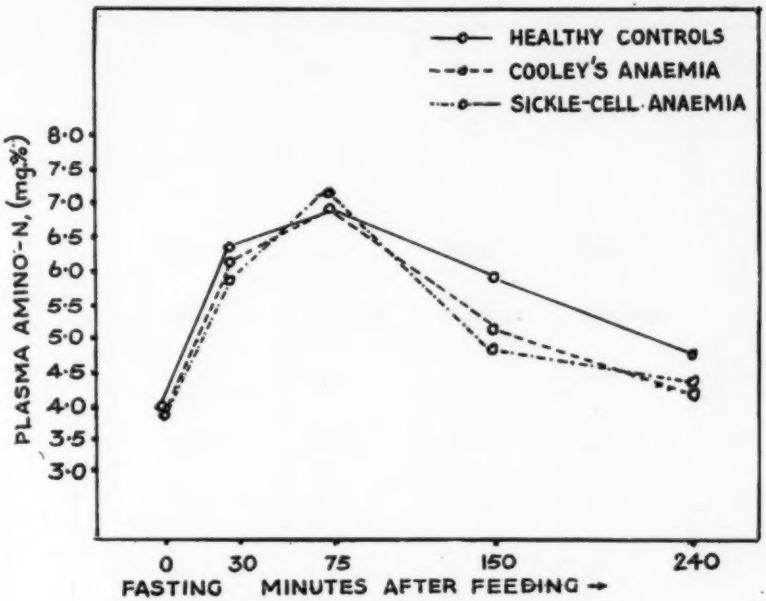

FIG. 1.—Mean amino-nitrogen curves after the ingestion of casein.

given by 100 μg. of taurine), corresponding to the fall in the plasma amino-N curve. Amino-aciduria appears to be more marked in Cooley's and sickle-cell anaemias.

From 150 to 240 minutes after the ingestion of the test meal the amino-aciduria decreases in normal controls (12–14 spots of amino-acids with colour intensity ranging between that given by 10 and 60 μg. of taurine), whereas patients with Cooley's and sickle-cell anaemias continue to excrete increased amounts of amino-acids (13–18 spots with colour intensity ranging between that given by 10 and 100 μg. of taurine).

Fig. 3 demonstrates the mean amino-nitrogen curves after the ingestion of protein hydrolysate.

As can be seen, all children showed a sharp rise in the plasma amino-N level 30 minutes after the test meal. In most cases the fasting level is exceeded by 100%. This elevation is consistent and more pronounced in the second post-prandial sample. No difference between the three groups was noted in the plasma amino-N levels 75 minutes after the ingestion of protein hydrolysate. The peak of the curve was found 75 minutes after the test meal.

The plasma amino-N level declines gradually after 75 to 150 minutes in healthy controls (still exceeding twice the fasting level), whereas in Cooley's or sickle-cell anaemias a sharp fall is noted.

It was finally noted that 240 minutes after the test meal the original fasting level is again approached in all the three groups. Values below the fasting level are designated as negative quantities.

Fig. 4 is a histogram of urinary amino-acid elimination after the ingestion of protein hydrolysate.

In healthy controls the most striking increase in urinary amino-acids occurs from 75 to 150 minutes from the test meal (13–17 spots of amino-acids, with colour intensity ranging between that given by 10 and 80 μg. of taurine); amino-aciduria continues beyond the 150 minutes, but to a lesser degree. On the other hand, in cases of Cooley's or sickle-cell anaemias, the greatest urinary output of amino-acids is between 0 and 75 minutes from the ingestion of the test meal (15–17 spots of amino-acids with colour intensity ranging from just above normal for considerably over the intensity given by 100 μg. of taurine); amino-aciduria continues beyond the 75 minutes and remains constantly increased, even after four hours.

Fig. 5 gives the mean amino-nitrogen curves after the intravenous administration of casein hydrolysate.

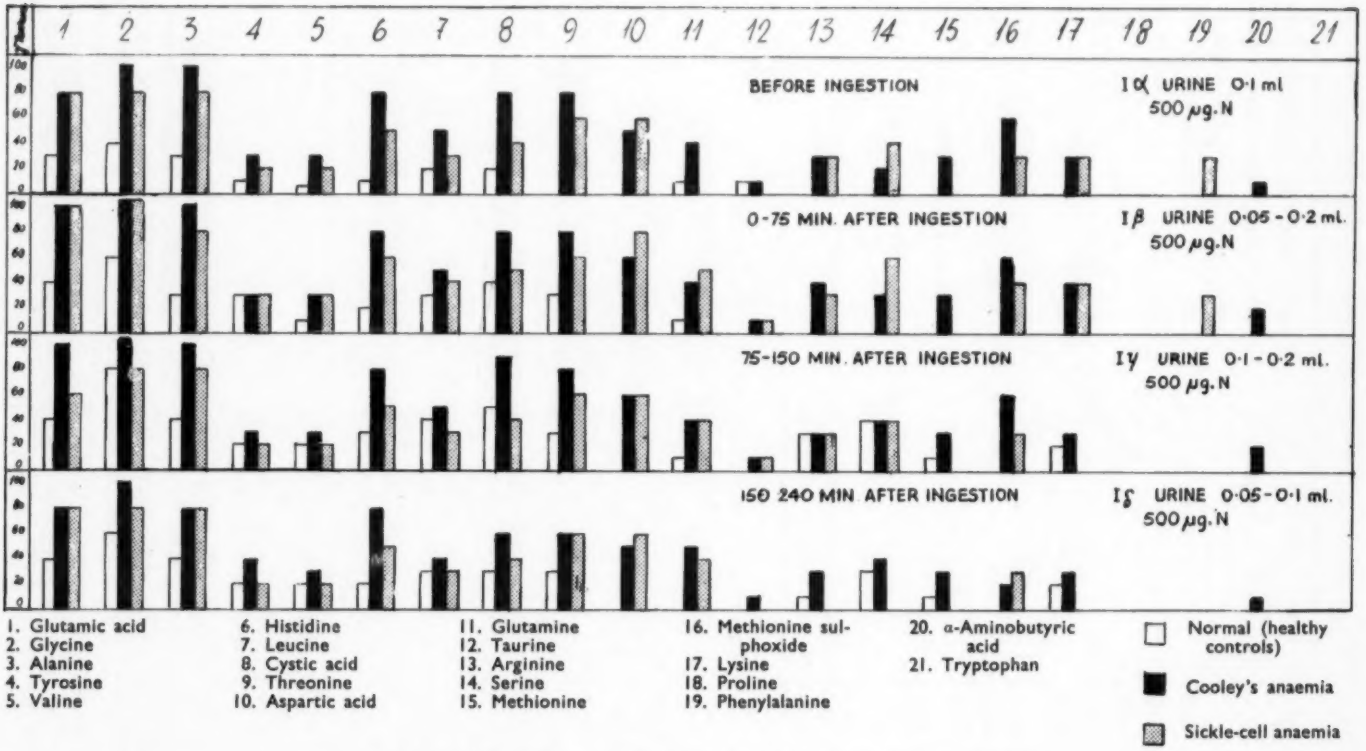

FIG. 2.—Histogram of elimination of amino-acids after the ingestion of protein (calcium caseinate).

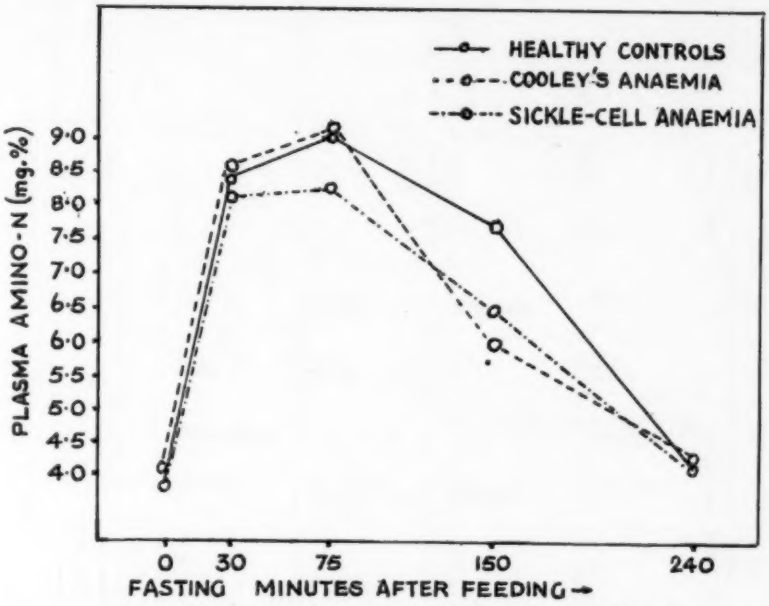

FIG. 3.—Mean amino-acid curves after the ingestion of casein hydrolysate.

As can be seen in all tested children, the amino-N level reaches its highest value at the completion of the injection of casein hydrolysate and then starts declining, so that in the five-minute sample the increase is 75% above the initial level. The decline continues progressively in healthy controls, whereas in patients with Cooley's or sickle-cell anaemias a sharp fall in the plasma amino-N level is noted.

Finally a return of the amino-acidaemia to fasting levels is noted in all the three groups after 90 minutes, in some instances to substantially lower than the fasting level, but this is not statistically significant. It is possible that this latter effect is referable to a synthetic process which requires some of the original amino-acid supply of the body (Harper, 1949).

Fig. 6 is a histogram of the elimination of urinary amino-acids after the injection of casein hydrolysate.

In healthy controls the highest degree of elimination was noted 15 minutes after the intravenous injection of casein hydrolysate (16–18 spots of amino-acids, with colour intensity ranging between that given by 10 and 90 μg. of taurine); amino-aciduria still continues even 90 minutes from injection but to a lesser degree.

In Cooley's and sickle-cell anaemia the highest degree of amino-acid elimination was sooner than that noted in the cases of normal children, occurring within 15 minutes from the injection of casein hydrolysate (15–18 spots of amino-acids, with colour intensity ranging from just above normal for considerably over the intensity given by 100 μg. of taurine). Amino-aciduria has already returned to the pathologically increased fasting levels in 90 minutes.

Discussion

Choremis and his associates (1957) showed that amino-aciduria without hyperamino-acidaemia is a constant finding in Cooley's and sickle-cell anaemia; it was also speculated that the amino-aciduria was due to a defective reabsorption of amino-acids in the renal tubules because of the possible damaging effect of haemosiderin.

The distinction between the metabolic and renal mechanism of amino-acidurias is very difficult. The most satisfactory way to prove the nature of an amino-aciduria is, according to Dent (1954), by estimating the individual amino-acid clearance. Since such a procedure could not be undertaken owing to technical difficulties, it was thought that the probable defective reabsorption of amino-

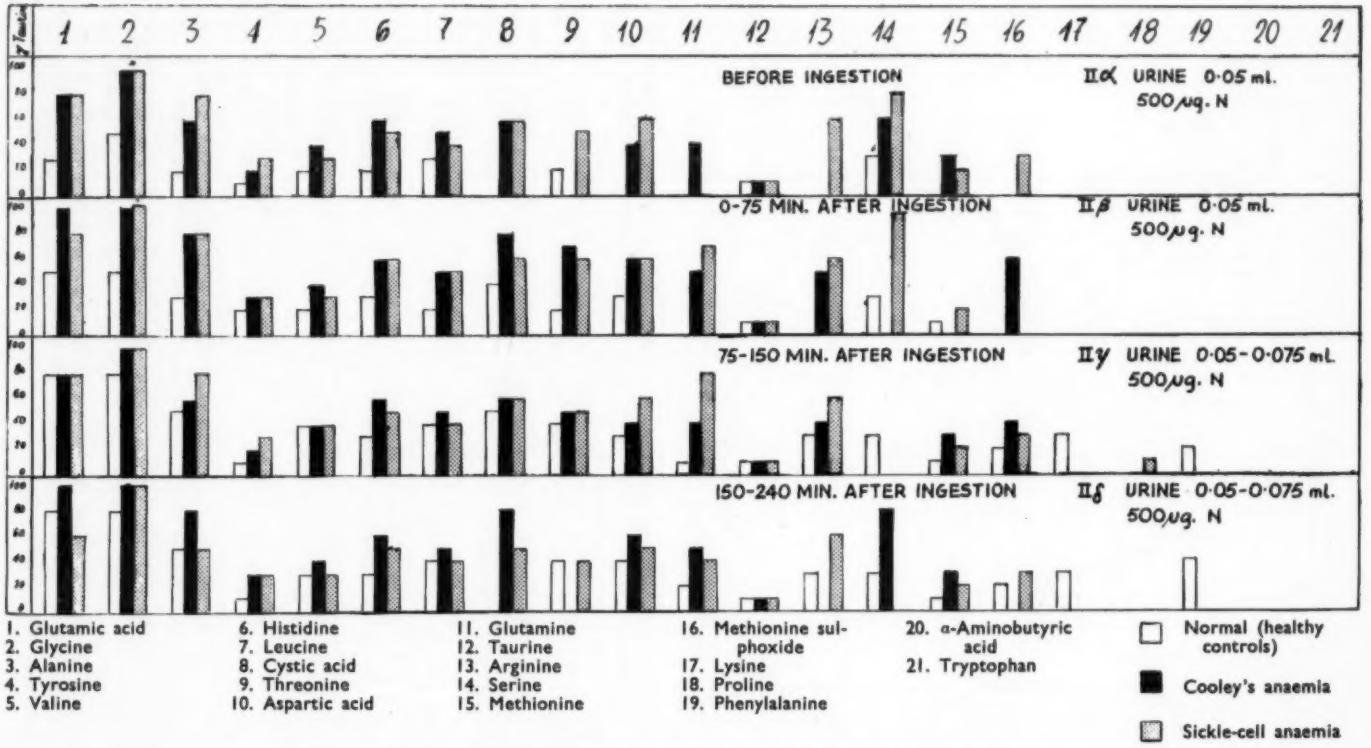

FIG. 4.—Histogram of the elimination of urinary amino-acids after the ingestion of casein hydrolysate.

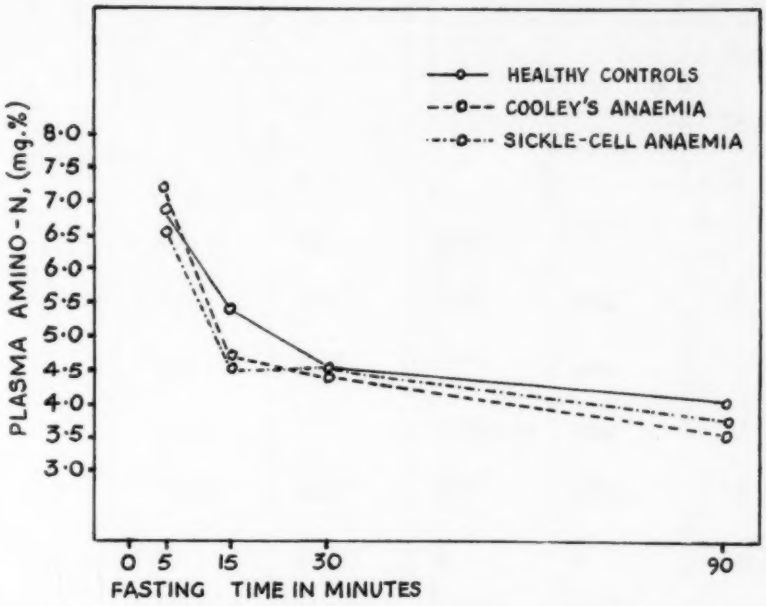

FIG. 5.—Mean amino-nitrogen curves after the intravenous administration of casein hydrolysate.

acids in the renal tubules could be studied as described above. By the same procedure, incidentally, it was possible to study both the proteolytic activity of the gastro-intestinal tract and the absorption of amino-acids. It was clearly shown that the latter functions were perfectly normal in patients with Cooley's and sickle-cell anaemia.

It is well known that when hyperaminoacidaemia occurs, especially after the rapid intravenous administration of amino-acids (Harper, 1949), some amino-acids escape the reabsorbing capacity of the renal tubules. After loading the patients of all three groups with oral calcium caseinate and protein hydrolysate and intravenous casein hydrolysate, amino-acids were excreted as expected; however, in the patients with Cooley's and sickle-cell anaemia the amino-aciduria was far more excessive (both quantitatively and qualitatively) than in the controls, indicating a possible defective reabsorption in the renal tubules. This excessive amino-aciduria was also reflected in the total plasma amino-N concentrations, which declined faster and more abruptly in the patients than in the controls, following both the oral and intravenous loading with protein and protein hydrolysate.

The "renal" versus the "overflow" nature of the amino-aciduria in such patients seems to be fairly certain; it is also quite probable that the increased glomerular filtrate (Papamattheaki, 1954) in patients with congenital haemolytic anaemias contributes little if anything to the degree of amino-aciduria, which is mainly due to a defective reabsorbing capacity of the renal tubules.

However, the exact mechanism of the amino-aciduria is not known; nevertheless it may be speculated that, as in most cases with congenital defects of the renal tubules, an enzymic dysfunction might be responsible for the amino-aciduria (Bickel, 1956; Fanconi, 1954, 1956; Jackson and Linder, 1953; Payne, 1956).

Conclusion

The increased elimination of amino-acids coupled with the abrupt fall of the plasma amino-N curve indicates that in Cooley's and sickle-cell anaemias the main error in handling amino-acids lies in their decreased kidney reabsorption. An enzymic dysfunction might be responsible.

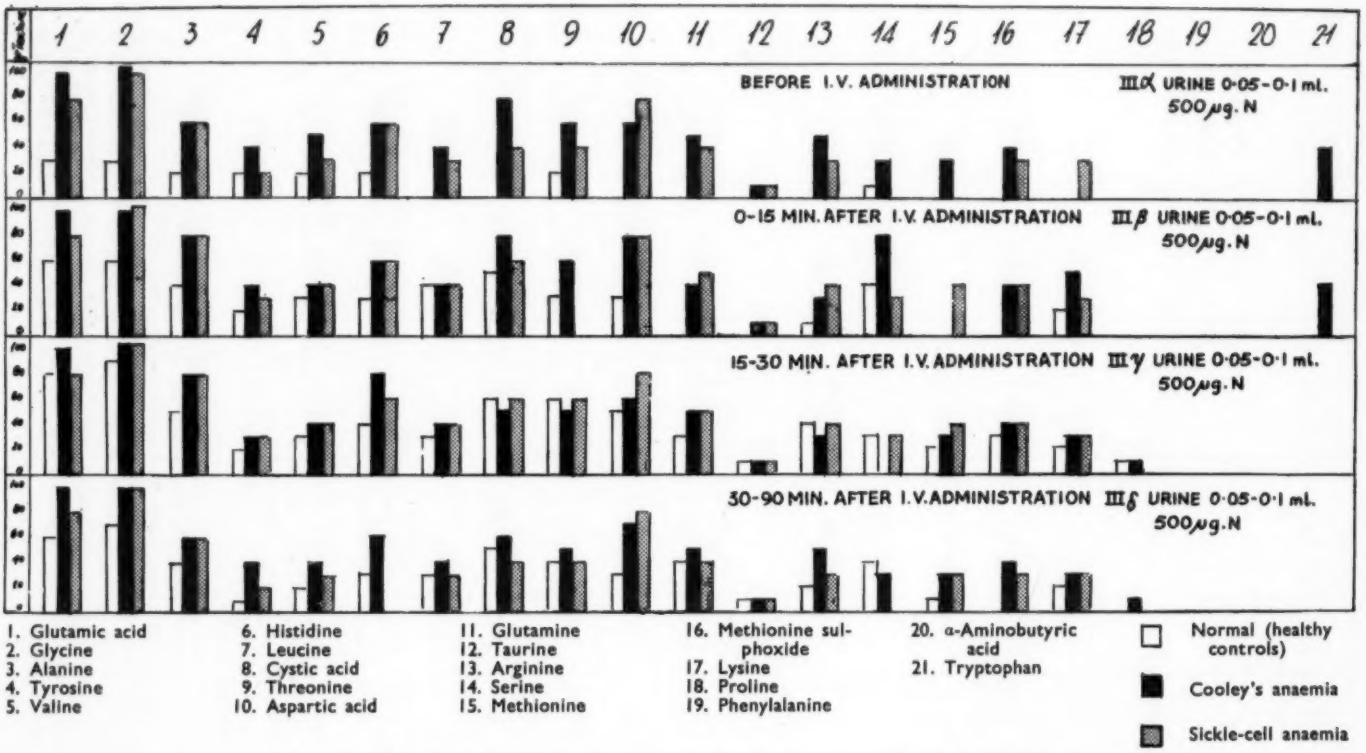

FIG. 6.—Histogram of the elimination of urinary amino-acids after the intravenous injection of casein hydrolysate.

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DILITHIUM SEQUESTRENE AS AN ANTICOAGULANT

BY

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The variety of different anticoagulants used for blood samples has increased, but there is still need for a suitable anticoagulant for the routine estimation of sodium and potassium by flame photometry. So far the only satisfactory substance for this purpose has been the expensive calcium heparin (King and Wootton, 1956).

Disodium sequestrene (Proescher, 1951 ; Hadley and Larson, 1953) and dipotassium sequestrene (Hadley and Weiss, 1955) are the most valuable of the recently introduced anticoagulants for routine haematological procedures because they preserve the morphology of leucocytes for short periods better than the ammonium and potassium oxalate mixture or heparin, and they prevent platelets clumping and preserve them. The disodium sequestrene is less satisfactory than the dipotassium salt because of its lower solubility in blood.

Dilithium sequestrene was prepared and its properties as an anticoagulant were investigated. It was found to have the properties of dipotassium sequestrene in routine haematological work but could also be used in many biochemical procedures, including flame photometry. This salt had previously been investigated (Hadley and Weiss, 1955) and found to be unsatisfactory because of its low solubility.

Preparation

Dilithium sequestrene can be prepared from sequestric acid by reaction with the theoretical amounts of either lithium carbonate or lithium hydroxide.

Method 1

$$\begin{array}{l} CH_2N\,(CH_2COOH)\,CH_2COOH \\ \quad | \qquad\qquad\qquad\qquad + Li_2CO_3 \\ CH_2N\,(CH_2COOH)\,CH_2COOH \\ \qquad 292.24 \qquad\qquad 73.89 \end{array}$$

$$\rightarrow \begin{array}{l} CH_2N\,(CH_2COOH)\,CH_2COOLi \\ \quad | \qquad\qquad\qquad\qquad .2H_2O + H_2O + CO_2 \\ CH_2N\,(CH_2COOH)\,CH_2COOLi \\ \qquad 340.14 \end{array}$$

Ethylene diamine tetra-acetic acid (E.D.T.A.), 29.2 g., and 7.4 g. of lithium carbonate were intimately mixed. Then 200 ml. of water was added and solution took place with vigorous evolution of carbon dioxide.

Method 2

E.D.T.A., 29.2 g., was dissolved in 200 ml. of normal lithium hydroxide solution (41.96 g. $LiOH.H_2O$ per litre).

In both cases the resulting solution contained the dilithium salt.

Dilithium sequestrene was obtained from these solutions after filtration to remove a small quantity of amorphous debris, by precipitation on the addition of an equal volume of absolute methyl alcohol and cooling. Dilithium sequestrene separated out as a fine white crystalline substance which was recovered by filtration on a buchner funnel and washed four times with 10 ml. of absolute methyl alcohol. The salt was dried at atmospheric pressure over anhydrous calcium chloride to a constant weight.

The usual yield by either of these methods was 29 g., namely, 85% of the theoretical yield.

Physical Properties

The substance produced by either method was identical. It was a white micro-crystalline solid which had a solubility in water of 16 g. per 100 ml. at 18° C. and was freely soluble in 10% v/v methyl alcohol in water but insoluble in absolute methyl alcohol. The melting point was 300° C. with decomposition. The theoretical lithium content of the dilithium salt is 4.08% and the estimated lithium content was 4.16% using the EEL flame photometer. This confirms that the dilithium salt has been produced.

Carbon and hydrogen estimations were performed on the dilithium sequestrene and the results obtained were hydrogen content 5.4% and carbon content 37.08%. The theoretical values for dilithium sequestrene with two molecules of water of crystallization is hydrogen 5.33% and carbon 35.3%.

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Distribution as an Anticoagulant

Distribution of dilithium sequestrene from aqueous solution was unsatisfactory as the deposit was difficult to dissolve in blood.

The most efficient method of distributing dilithium sequestrene was by placing the appropriate amount of a solution containing 2.1 g. per 100 ml. in 10% v/v methyl alcohol in water into each universal container. These containers were placed in a hot air oven at 160° C. for 30 minutes. This procedure did not affect the anticoagulant properties of the salt or in any way lower its solubility in blood or cause its decomposition. All traces of methyl alcohol were also removed.

The optimal amount of dilithium sequestrene for routine haematological and chemical estimations was found to be 0.2 ml. of this solution for 5 ml. of blood, i.e., 0.84 mg. per ml. The minimum amount to prevent coagulation is 0.52 mg. per ml.

The tolerance for haematological procedures, which ensures retention of good cell morphology, is between 1.05 mg. and 0.66 mg. per ml. of blood.

The tolerance for blood urea and electrolytes, except plasma chloride, is between the minimum amount necessary to prevent coagulation, i.e., 0.52 mg., and 4.2 mg. per ml. of blood.

For chlorides between 0.52 and 1.4 mg. per ml. is satisfactory.

Dipotassium and disodium sequestrene have been distributed into universal containers using the method previously described. The solubility of these salts in blood was as good as the solubility of dilithium salt under similar conditions.

Comparative chemical estimations were made on samples of the same blood taken into dilithium sequestrene and calcium heparin.

Comparative haematological investigations were made on samples of the same blood taken into dilithium sequestrene, dipotassium sequestrene, and the ammonium and potassium oxalate mixture.

Results

Chemical Investigations.—The wavelength of the emission spectrum for lithium is 6708 Å, for sodium 5890 Å and 5896 Å, and for potassium 7655 Å. Using the EEL flame photometer with a coal gas/air flame no interference was detected due to lithium on the intensity of the sodium or potassium emission spectra, even when using 10 times the amount of lithium sequestrene normally present.

Sodium and potassium were estimated, using the flame photometer, on plasma from samples of blood anticoagulated with either calcium heparin or dilithium sequestrene. Plasma chloride was estimated by the method of Schales and Schales (1941).

Blood urea was estimated by the method of Archer and Robb (1925) and plasma bicarbonate in the volumetric Van Slyke apparatus.

The electrolyte results obtained from 100 comparable samples of plasma are tabulated in Table I.

TABLE I
COMPARABLE ELECTROLYTE RESULTS

	Range of Variation (mEq. per Litre)			
	±2	±4	>±4	Mean Error
Sodium	75	25	0	+0·04
Chloride	84	15	1	+1
Bicarbonate ..	99	1	0	+0·08
	mEq. per litre			
	±0·2	±0·4	>±0·4	
Potassium	95	4	1	−0·015

Of the 100 comparative urea estimations, 99 were within 10% of the calcium heparin standard and one only was outside this range. The figures in this case were 43 mg. per 100 ml. for the calcium heparin and 38 mg. for the sequestrene estimation.

Sequestrene plasma was also found to be equally effective for the estimations of bilirubin, cholesterol, and the phosphatases.

Blood sugar estimations performed by either the Hagedorn and Jensen or Folin and Wu methods were unsatisfactory when compared with blood containing fluoride, as there was a rapid loss of reducing substance. It was, however, noted that the loss in the first one and a half hours after collection was about 5%, so that estimations performed on blood within this time or when fluoride was added within this time were satisfactory.

Haematological Investigations.—In the investigation of dilithium sequestrene as an anticoagulant for haematological investigations a comparison was made between samples of the same blood taken into the ammonium and potassium oxalate mixture, which were estimated after contact with the anticoagulant for one hour, and dipotassium sequestrene and dilithium sequestrene, which were estimated after one and four hours' contact with the anticoagulant.

On each sample of blood taken in the ammonium and potassium oxalate mixture after one hour and each sample in dipotassium or

dilithium sequestrene after one and four hours the following investigations were performed:

(1) Haemoglobin using the oxyhaemoglobin method reading in a photo-electric colorimeter using an Ilford 625 filter, (2) blood sedimentation rate (Wintrobe method); (3) packed cell volume using an M.S.E. haematocrit centrifuge governed to 3,000 r.p.m. for 30 minutes; (4) platelet count using direct counting in blood diluted either 1 in 20 in Barr's fluid or 1 in 100 in formol citrate (Dacie, 1956); (5) total white cell count; (6) blood films had differential white cell counts performed and were also examined for red cell morphology, platelet clumping and morphology, and the effect of the anticoagulants on leucocyte morphology.

Increased red cell destruction after contact with each of the three anticoagulants used was not found, as shown by spectroscopic examination of the supernatant plasma, plasma haemoglobin estimations, or the potassium level of separated plasma.

The results obtained by these methods on 50 different samples of blood are summarized.

(1) Haemoglobin Levels.—The maximum difference between all the five haemoglobin estimations on one samp'e of blood was less than 10% in all specimens and less than 5% in 60% of the specimens.

(2) The Sedimentation Rate.—A Wintrobe technique blood sedimentation rate was performed on each sample of blood after one and four hours' contact with each of the three anticoagulants used.

Seventy-six per cent. of these groups of samples gave readings within 2% of each other when all three anticoagulants were compared at one and at four hours' contact.

The remaining 24% gave identical readings at one hour but showed marked and erratic increase of the sedimentation rate after four hours' contact with the anticoagulant.

(3) Packed Cell Volume.—Generally there was less than 1% difference on any sample of the same specimen, but in eight specimens (16%) a constant reduction of 1% was noted in those samples which had been in contact with the dipotassium or dilithium sequestrene, or ammonium and potassium oxalate anticoagulant for four hours when compared with a contact time of one hour. This is suggestive of a minor degree of red cell shrinkage due to the anticoagulant.

(4) Platelet Count.—Platelet counts on the blood taken in the ammonium and potassium oxalate mixture were unsatisfactory as in all samples platelets had clumped.

Platelet counts on sequestrene blood were satisfactory. All duplicate platelet counts varied within a range of $\pm 10\%$, which is satisfactory.

It was noted occasionally that the platelets clumped in dilithium sequestrene if the anticoagulant had not been heated correctly at 160° C. for 30 minutes.

(5) White Cell Count.—The maximum variation noted in wet counts was well within 20% limits (Dacie, 1956).

(6) Blood Films.—(*a*) No significant difference in the differential white cell counts was noted between the ammonium and potassium oxalate anticoagulant after one hour and dipotassium and dilithium sequestrene after one and four hours' contact time.

(*b*) Red cell morphology appeared to be unaffected by the anticoagulants used.

(*c*) Platelets were clumped, usually into small clumps with a few free platelets, by the ammonium and potassium oxalate anticoagulant after one hour's contact.

Platelets remained free, evenly distributed throughout the films and of good morphology with either of the sequestrene anticoagulants.

(*d*) *Leucocyte Morphology.*—Nearly all films from blood in ammonium and potassium oxalate anticoagulant showed changes in the leucocytes due to anticoagulants. Most films showed between 4% and 10% of white cells affected by this anticoagulant after one hour's contact.

Most films made from either sequestrene anticoagulant after one hour's contact failed to show any loss of morphological characteristics.

After four hours' contact with either of the sequestrene anticoagulants 60% of films showed no loss of morphological characteristics in the white cells, 34% showed between 1% and 2% of white cells affected, and 6% showed from 3% to 5% of white cells affected.

Dilithium sequestrene is not satisfactory for the estimation of prothrombin time.

Conclusion

Dilithium sequestrene is as good an anticoagulant for standard haematological investigations as dipotassium sequestrene.

Dilithium sequestrene is a good anticoagulant for many of those chemical investigations normally performed on serum, such as sodium, potassium, chloride, bicarbonate, urea, phosphatases, cholesterol, and bilirubin. This is particularly important with the electrolytes, as it

enables rapid separation of the plasma, more accurate results due to this rapid separation, and less delay when these investigations are performed as emergency procedures.

Dilithium sequestrene is easy to produce and distribute and the cost is low. The reagents to make 30 g. of this salt cost approximately 2s. 6d.

Summary

The preparation and distribution of dilithium sequestrene, which may be used as a universal laboratory anticoagulant, are described.

This anticoagulant and the more usual laboratory anticoagulants for haematological and biochemical procedures are compared.

We wish to thank Dr. E. N. Allott and Dr. J. C. Thompson for their advice and co-operation during this investigation, and Miss P. A. Wyatt and Mr. A. P. Reynolds for their technical assistance.

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THE USE OF A SEQUESTRENE-CITRATE MIXTURE IN THE ESTIMATION OF THE BLOOD SEDIMENTATION RATE

BY

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Many methods have been designed for estimating the erythrocyte sedimentation rate (E.S.R.), but the method of Westergren (1921) seems now to be the most widely used (Dacie, 1956). It has been preferred by several workers (Davis, 1946; Gilmour and Sykes, 1951; Goldberg and Conway, 1952; Goldberg, Glynn, and Bywaters, 1952) because it was felt to reflect the changes due to disease more accurately than the other methods, notably that of Wintrobe and Landsberg (1935). This preference has been endorsed recently by Galbraith and Jones (1958), who showed that high sedimentation rates could be found with the Westergren technique when the Wintrobe E.S.R. was normal or only slightly raised.

The only practical difficulty in estimating the E.S.R. has so far been the need to set up the sedimentation tubes within a fairly short time of withdrawing the blood. Many workers have observed that allowing the blood to stand, even for a few hours, is attended by progressive retardation of the rate of sedimentation (Boerner and Flippin, 1935; Cooper, 1926; Dorfman and Brooks, 1937; Gilligan and Ernstene, 1934; Ham and Curtis, 1938). Nichols (1942) stated that the general consensus of opinion was that delay in measuring the sedimentation of a sample might cause retardation of the rate. The maximum amount of permissible delay reported was variable, from a "few" to "several" hours. Wintrobe, describing his own method in 1951, stated that the blood should be used within two hours of collection for the determination of the E.S.R. Hadley and Weiss (1955) noted that the E.S.R. of stored oxalated blood was obviously unreliable.

One exception to this general retardation of the E.S.R. was pointed out by Koster (1937), who observed in cases of carcinoma and lymphogranuloma that the E.S.R. after blood had been stored for 24 hours showed no change. This was confirmed by Feldman (1940).

Nichols (1942) suggested that the retarding effect of delay in setting up the blood for measuring the E.S.R. might bear some relationship to the kind of anticoagulant used, and also to the temperature at which the blood samples were kept.

Present Investigation

In view of Nichols' suggestion, it was decided to investigate the sedimentation rate of both fresh and stored blood, using a modified Westergren technique incorporating sequestrene as the anticoagulant. Sequestrene (the disodium salt of ethylenediamine tetra-acetic acid) was first described by Dyckerhoff, Marx, and Ludwig (1942). Its use in routine haematology was described by Proescher (1951), Hadley and Larson (1953), Schmidt, Hane, and Gomez (1953), Wittgenstein (1953), and Hadley and Weiss (1955). As an anticoagulant it is 10 times more powerful than sodium citrate, but its most notable feature is its capacity to preserve the blood elements for several days. Schmidt *et al.* considered that sequestrene was superior to both heparin and oxalate for routine work. It has the added advantage of being very cheap.

Methods

Comparison of E.S.R. of Fresh Blood Using Westergren and Modified Sequestrene-citrate Techniques.—Twenty-five samples of venous blood were withdrawn for study from 22 patients who were being treated or investigated in a medical unit of the Royal Infirmary, Glasgow. Four millilitres of each sample was added to 1 ml. of 3.8% sodium citrate solution in a graduated test tube (tube A), and 5 ml. was placed in a bijou bottle containing 5 mg. of dried sequestrene. As soon as possible after the withdrawal of the blood, and in no case later than two hours after venepuncture, the sequestrene blood was mixed

by manual inversion and rotation for three minutes (except where stated this mixing method was used throughout this work) and 4 ml. of this blood was added to another test tube containing 1 ml. of the sodium citrate solution (tube B). The contents of tube A were then mixed, and a pair of Westergren E.S.R. tubes filled from it and set up in the E.S.R. rack. The same procedure was carried out with the contents of tube B. The four sedimentation rates were read by one of us after one hour.

The rack was at first fitted with two spirit levels to ensure that the E.S.R. tubes were vertical. Later this device was abandoned in favour of a simple plumb line hanging from the top bar of the rack.

Comparison of E.S.R. of Fresh Blood and of Blood Stored for 24 Hours Using Sequestrene-citrate Technique.—Twenty-five 10 ml. samples of venous blood were collected and added to bottles containing 10 mg. of dried sequestrene. After mixing, 4 ml. blood was taken from each sample and diluted with 1 ml. of 3.8% sodium citrate solution in a test tube. The contents of the test tube were mixed and two Westergren E.S.R. tubes filled from it and set up. The sedimentation rates were read after one hour.

The remainder of each original blood sample was stored in the refrigerator at temperatures varying between 5 and 10° C. After about 24 hours the stored blood was mixed by rotation in a mechanical mixer (Matthews, 1951) for at least 10 minutes. Four millilitres was then added to 1 ml. of the sodium citrate solution, mixed, and replicate estimations of the E.S.R. carried out as above. The stored blood should have reached room temperature by the time the dilution and mixing had been completed and temperature measurements were not made.

TABLE I

E.S.R. OF FRESH BLOOD USING CLASSIC WESTERGREN AND MODIFIED SEQUESTRENE-CITRATE METHODS

Sample No.	Sex	Age	Diagnosis	E.S.R. Citrate Only		E.S.R. Sequestrene-citrate	
				mm.	mm.	mm.	mm.
1	M	38	Anaemia	3	2·5	2·5	3
2	F	59	Bronchitis ..	12	11	9·5	12
3	F	51	Thyrotoxicosis ..	6	6	6	6
4	F	77	Hemiplegia ..	7	7	7	8
5	F	24	Coarctation of aorta	6	6	6	6
6	F	62	Rheumatoid arthritis	88·5	83	95	96
7	M	55	Pulmonary infarct	58	50	57	57
8	M	76	Lung abscess ..	101	98	99	96
9	F	61	Myocardial infarct	25	25	26	29
10	F	60	,, ,,	10	10	10	10
11	F	29	Rheumatism ..	68	70	63	65
12	F	61	Pulmonary infarct	40	43	51	48
13	M	62	Myocardial ,,	40	40	39	39
14	M	63	Hypertension ..	2·5	4	4	4
15	M	68	Bedsores	105	103	103	107
16	M	55	Pulmonary infarct	24	26	27	24
17	F	24	Hypersplenism ..	4	4	4	4
18	F	48	Pneumonia ..	4	4	4	4
19	F	61	Myocardial infarct	13	12·5	11	11·5
20	M	57	Duodenal ulcer ..	96	92	97	98
21	M	47	Atheroma ..	6	7	6	7·5
22	F	29	Rheumatism ..	48	48	46	47
23	F	61	Pulmonary infarct	39	40·5	43	39
24	F	65	Pernicious anaemia	5·5	5·5	5·5	5·5
25	F	74	Myocardial infarct	18	18	20	18
Mean value of 25 estimations ..				33·18	32·64	33·66	33·78
,, ,, ,, 50 ,, ..				32·91		33·72	

Results

The results of the experiments are shown in Tables I and II, which give the age, sex, and diagnosis of each case. Mean values for the E.S.R. are also shown.

Discussion

The results have been submitted to analysis of variance and this has shown that there is no significant difference between the mean values 32.91 and 33.72 in experiment 1, or between the mean values 32.27 and 32.08 in experiment 2.

With the classic Westergren technique in experiment 1 the maximum differences between duplicate sedimentation rates were 8 mm. in sample No. 7 and 5.5 mm. in sample No. 6. In 20 of the 25 samples the difference between the duplicates was 2 mm. or less. With the modified technique in experiment 1, 18 of the samples showed a difference of 2 mm. or less, five a difference of 2 to 3 mm., and the greatest difference was of 4 mm. in sample No. 15 (range 103–107 mm.) and in sample No. 23 (range 39–43 mm.).

The maximum discrepancy between pairs of sedimentation rates from the same patient was seen in sample No. 6 (difference of means 9.75 mm. in a range of 83 to 96 mm.). Nineteen samples showed a difference of means of 2 mm. or less, and two a difference of means of 2 to 3 mm.

The results were so closely comparable that we accepted that the sequestrene-citrate modification provided an accurate method of estimating the Westergren E.S.R.

From Table II it is seen that in 23 samples the difference between duplicate sedimentation rates of fresh sequestrene-citrate blood was 2 mm. or less. The greatest difference was 4 mm. in sample No. 42 (range of 58.5 to 62.5 mm.). The duplicate estimations on the stored sequestrene blood showed no difference greater than 2 mm. The maximum differences between means of sedimentation rates of fresh blood and stored blood are shown in Table III.

In 15 of the samples in experiment 2 the difference of the mean E.S.R. of fresh and stored blood was 2 mm. or less, in four samples it lay between 2 and 3 mm., and in one sample it was 4 mm. Despite the close comparability of these 20 results, and despite the satisfactory analysis of variance, some further consideration must be given

TABLE II
E.S.R. OF FRESH BLOOD AND OF BLOOD STORED FOR 24 HOURS USING SEQUESTRENE-CITRATE TECHNIQUE

Sample No.	Sex	Age	Diagnosis	E.S.R. Fresh Blood		E.S.R. Stored Blood	
				mm.	mm.	mm.	mm.
26	M	52	Pneumonia ..	32	31·5	33·5	33
27	M	62	Myocardial infarct	4	4·5	3·5	4
28	M	52	Thrombophlebitis	4	4·5	3	3
29	M	63	Carcinoma ..	34	33	26	26
30	M	47	Hypertension ..	9	9	8	8
31	M	41	Mitral valvotomy ..	7	6·5	7	7
32	M	47	Gastro-enterostomy	36·5	33	35	34
33	M	73	Pneumonia ..	109	109	112	111
34	M	67	Myocardial infarct	12	12	10	11
35	M	42	Lupus erythematosus	6	7	8·5	9
36	M	67	Polycythaemia ..	1	1	0·5	1
37	F	61	Myocardial infarct	13	13	11·5	12
38	F	36	Anaemia	4	4	4	3·5
39	F	59	Thrombophlebitis	26·5	24·5	20	19
40	F	70	Rheumatoid arthritis ..	100	98	121	120
41	F	52	Polyserositis ..	19	19	18·5	17
42	F	52	Rheumatoid arthritis ..	62·5	58·5	63·5	62·5
43	F	73	Pernicious anaemia	26	28	26	27
44	F	35	Hepatitis	138	139	133	131
45	F	60	Carcinomatosis ..	17	16	17·5	17
46	F	60	Rheumatoid arthritis ..	22	22	16	15
47	M	86	Urine infection ..	21	20	19	19
48	F	45	Rheumatoid arthritis	32	34	38	36
49	F	43	,, ,,	29	28	28	26
50	F	55	Cellulitis	47	47	44	45
Mean value of 25 estimations ..				32·46	32·08	32·28	31·88
,, ,, ,, 50 ,, ...				32·27		32·08	

TABLE III
DIFFERENCES OF MEANS OF SEDIMENTATION RATES OF FRESH BLOOD AND OF BLOOD STORED FOR 24 HOURS

Sample No.	Difference of Means (mm.)	Range of E.S.R. (mm.)
40	21·5	98 to 121
29	7·5	26 ,, 34
46	6·5	15 ,, 22
44	6·5	131 ,, 139
39	6·0	19 ,, 26·5

to the discrepancies listed in Table III. What clinical significance is to be attached to them? If the E.S.R. of the fresh blood sample is taken as the "correct" E.S.R., it will be seen from Table II that storage of the blood was followed by acceleration of the sedimentation rate in sample 40 but by slight retardation in samples 29, 46, 44, and 39. It is thought safe to disregard the retardation in sample No. 44 in which the rate fell from a mean value of 138.5 to a mean value of 132 mm. In none of the other three cases of retardation has the E.S.R. change any clinical significance, whether the sedimentation test is used as a screening procedure or as an observation on the progress of a case. We think that our results compare favourably with those of Goldberg and Conway (1952), who modified the Westergren method by the addition of oxalated blood to 3.8% sodium citrate solution. They showed that, with the modified technique, the E.S.R. of oxalated blood was quite inaccurate after storage for 24 hours at room temperature.

There are only a few reports of the use of sequestrene in estimating the blood sedimentation rate, and these describe Wintrobe techniques or do not specify the E.S.R. technique used. A comparison was made by Hadley and Larson (1953) of the E.S.R. of blood, some of which had been treated with oxalate and the rest with sequestrene. They found no significant differences in 26 comparisons. Proescher (1951) had observed that if sequestrene blood was stored at 5° C. the E.S.R. remained about the same for several days provided that the blood was re-mixed without excessive shaking. Hadley and Weiss (1955) stored sequestrene blood for 24 hours and noted that the E.S.R. was considerably less affected by the storage than was the E.S.R. of stored oxalated blood.

In the present investigation, with a modified Westergren technique, it has been shown that the storage of sequestrene blood for 24 hours at 5–10° C. had no effect on the E.S.R. of 20 out of 25 samples. The storage was followed by a clinically insignificant slowing in four samples and by a moderate acceleration in the remaining one.

The introduction of the sequestrene-citrate method, therefore, obviates the need to dilute blood in sodium citrate at the bedside, as in the standard Westergren method, and the E.S.R. can be reliably estimated after a blood sample has been stored for 24 hours. The modified method would be of particular value in hospital out-patient departments, or possibly as part of a clinical laboratory service for general practitioners. It will be noted in passing that a complete blood examination, including an E.S.R., can be carried out on one sample of sequestrene blood.

Summary

The retardation of the E.S.R. which is seen when blood is stored is thought to be related to the anticoagulant used and to the temperature at which it is stored. Sequestrene, a recently introduced anticoagulant, has been used in a study of the E.S.R. of fresh blood and of blood stored for 24 hours at 5 to 10°, using a modified Westergren technique. The E.S.R. of blood treated with sequestrene and then added to sodium citrate solution does not differ from the classic Westergren E.S.R. Storage of sequestrene blood as outlined above makes no appreciable difference to the E.S.R. using the modified technique. The

introduction of the sequestrene-citrate method will extend the use of the sedimentation test in clinical practice.

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THE EFFECT OF SEQUESTRENE AND HEPARIN ON THE ERYTHROCYTE SEDIMENTATION RATE

BY

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The Westergren method for the determination of the erythrocyte sedimentation rate has been conclusively shown to be more reliable than other methods (Gilmour and Sykes, 1951; Goldberg, Glynn, and Bywaters, 1952; Lewi and Clarke, 1954; Alexander and Andrews, 1957; Eastham, Denson, and Evans, 1958).

Blood for a variety of haematological and biochemical investigations is commonly collected into Wintrobe oxalate mixture, heparin, or sequestrene (ethylenediamine tetra-acetic acid), as anticoagulant. The use of such blood samples for determining the erythrocyte sedimentation rate obviates the necessity of collecting a separate specimen.

Rourke and Plass (1929) have shown by the addition of heparin to haemophilic blood that heparin itself has no effect on sedimentation when used at a concentration of 20 units/ml. but that an excessive amount increases the sedimentation rate. Both heparin and oxalate when used as anticoagulants in determining the E.S.R. have been shown to give anomalously low one-hour readings. These low readings have been abolished and greatly increased readings obtained by the addition of 3.8% sodium citrate as used in the standard Westergren method (Goldberg *et al.*, 1952; Lewi and Clarke, 1954). In these cases the addition of sodium citrate produces three effects: (1) Lowering of the packed cell volume by simple dilution; (2) further lowering of the P.C.V. due to cell shrinkage by the hypertonic sodium citrate; (3) dilution of the plasma proteins.

Effects (1) and (2) increase the sedimentation rate since this is inversely proportional to the P.C.V. (Meyers, Trevorrow, Washburn, and Mugrage, 1953; Lawrence, 1953) and outweigh the effect of (3) which decreases the sedimentation rate (Fåhraeus, 1929; Lewi and Clarke, 1954).

The effect of sequestrene on erythrocyte sedimentation has not been investigated. Ham and Curtis (1938) and Mårtensson and Hansen (1953) have demonstrated that the addition of oxalate mixture to citrated blood has no effect on the E.S.R. The present investigation was undertaken to evaluate the effect of sequestrene on erythrocyte sedimentation, and to compare the effect of the addition of 3.8% sodium citrate to blood which had been collected into heparin or sequestrene with citrated blood as used in the standard Westergren method.

Methods

Twenty millilitres of blood was obtained by venepuncture using a wide bore needle, and quickly distributed into calibrated tubes in the following manner:

(1) 8 ml. into 16 mg. of sequestrene (dry sodium salt) to give a final concentration of 2 mg./ml.

(2) 8 ml. into 160 units of dry heparin to give a final concentration of 20 units/ml.

(3) 4 ml. into 1 ml. of 3.8% trisodium citrate.

One millilitre of 3.8% sodium citrate was then added to 4 ml. of the heparinized and sequestrenized blood and the samples containing sequestrene, sequestrene+citrate, heparin+citrate, and citrate allowed to stand at room temperature for half an hour. After this time they were thoroughly mixed, and, using standard Westergren sedimentation pipettes of 2.5 mm. bore, 200 mm. columns of blood from each sample were set up in triplicate in sedimentation racks at room temperature. The separate samples were quickly mixed between each set of triplicate tubes and the time of insertion of each tube noted in order to ensure that readings were made at exactly 60 minutes.

The heights of the supernatant plasma columns were measured at three-minute intervals for a period of 60 minutes, and from the readings graphs of time against plasma height were plotted, and the maximum sedimentation rates obtained (240). The same set of tubes and stands were used throughout the series.

Blood samples were obtained from patients in whom a raised sedimentation rate was anticipated. Because of this method of selection and the inclusion of extreme cases, together with the small sample size, a few values were rejected as outliers for the purpose of the calculations, since it was felt that a simple analysis would be misleading.

For rejections in Tables I and II Cochran's test was applied ($P<1\%$).

For rejections in Table III the test statistic $q=\frac{w}{s}$ ($P<1\%$) was applied.

Results

Tables I and II give the 20 estimates of the variance (2 degrees of freedom mean squares) of the 60 minute readings and maximum sedimentation rates for the four anticoagulant series. These are given in their ranked orders of increasing size and clearly are not homogeneous. A few significantly large variance estimates have been rejected as outliers whenever they form more than a reasonable fraction of the total for the group (Cochran, 1941 ; Eisenhart and Solomon, 1947). These rejections do not lead to any substantial differences for the average variance estimates between the series as shown in Tables I and II. The standard deviations for citrate + heparin, citrate + sequestrene, and citrate agree well as a group and with previous estimates of the standard deviation for citrate + oxalate (Eastham *et al.*, 1958). However, the standard deviation for sequestrene is significantly greater in comparison, and indicates a high degree of variability inherent in the use of sequestrene alone, as has been previously shown for potassium/ammonium oxalate mixture and heparin.

TABLE I

MEAN SQUARES OF DIFFERENCES FOR TRIPLICATE E.S.R. DETERMINATIONS FOR EACH ANTICOAGULANT SERIES

One Hour Readings				
	Sequestrene	Citrate	Sequestrene + Citrate	Heparin + Citrate
	0·00	0·00	0·00	0·00
	0·22	0·00	0·00	0·00
	0·89	0·00	0·00	0·00
	1·56	0·00	0·00	0·00
	1·56	0·22	0·00	0·00
	1·56	0·22	0·00	0·00
	2·00	0·22	0·00	0·00
	4·67	0·22	0·00	0·22
	8·00	0·22	0·22	0·22
	8·22	0·22	0·22	0·67
	21·56	0·22	0·22	0·89
	24·00	0·67	0·22	0·89
	29·56	0·67	0·67	1·56
	33·89	0·67	0·67	1·56
	38·89	0·67	0·67	1·56
	53·54	0·89	1·56	2·00
	83·67	0·89	2·00	3·56
	128·00	2·00	4·22 R	4·67
	141·55	2·67	5·56 R	6·89
	1,689·92 R	3·56	108·67 R	10·89
Average mean square	30·7	0·71	0·38	1·78
S.D.	5·54	0·84	0·62	1·33
D.F.	38	40	34	40
S.D. 20 cases	10·66	0·84	2·50	1·33
D.F.	40	40	40	40

N.B. Each mean square has 2 degrees of freedom. R = Rejected by outlier test.

TABLE II

MEAN SQUARES OF DIFFERENCES FOR TRIPLICATE E.S.R. DETERMINATIONS FOR EACH ANTICOAGULANT SERIES

Maximum Sedimentation Rates				
	Sequestrene	Citrate	Sequestrene + Citrate	Heparin + Citrate
	0·00	0·00	0·00	0·00
	0·00	0·00	0·00	0·00
	0·00	0·00	0·00	0·00
	0·002	0·00	0·00	0·00
	0·007	0·00	0·00	0·00
	0·016	0·00	0·00	0·00
	0·024	0·002	0·00	0·002
	0·027	0·002	0·00	0·002
	0·029	0·002	0·002	0·002
	0·029	0·002	0·002	0·002
	0·029	0·002	0·002	0·002
	0·036	0·002	0·002	0·002
	0·043	0·002	0·002	0·002
	0·058	0·002	0·002	0·003
	0·122	0·002	0·003	0·007
	0·142	0·003	0·003	0·007
	0·149	0·009	0·003	0·009
	0·363	0·016	0·009	0·021
	0·746	0·016	0·020 R	0·096 R
	0·802	0·016	0·629 R	0·229 R
Average mean square	0·131	0·0039	0·0017	0·0034
S.D.	0·362	0·063	0·041	0·058
D.F.	40	40	36	36

N.B. Each mean square has 2 degrees of freedom. R = Rejected by outlier test.

TABLE III

COMPARISON OF ARITHMETIC MEANS OF TRIPLICATE E.S.R.s IN THREE ANTICOAGULANT MIXTURES WITH THOSE USING CITRATE ALONE

One Hour Readings Showing Difference from Citrate (mm.)			
Sequestrene	Sequestrene + Citrate	Heparin + Citrate
−80·33 R	−20·67 R	−0·33
−41·67 R	−2·00	−0·33
−23·00 R	−2·00	1·00
−9·33	−1·67	1·00
−0·33	−0·67	1·33
1·00	0·00	1·67
1·33	0·00	2·00
1·67	0·33	2·67
2·33	1·33	2·67
7·00	2·33	3·33
10·33	2·67	4·00
10·67	2·67	4·33
11·00	3·67	4·33
11·33	4·00	6·00
14·00	4·33	6·67
16·00	6·00	6·67
17·16	6·00	8·00
19·67	6·33	10·00
21·33	9·33	10·00
26·33 R	19·33 R	16·33
Arithmetic mean +8·45	+2·37	+4·53
S.D. 8·22	3·15	4·58
*Coefficient of variance 13·0	5·0	7·3
S.D. (20 cases) 24·71	7·24	4·58

N.B. * This is not Pearson's coefficient of variation, but is obtained by expressing as a percentage the S.D. divided by the average of the 20 arithmetic means for the citrate readings.
R = Rejected by outlier test.

In Table III the differences between the arithmetic means of the triplicate citrated samples and the triplicate samples of the remaining anticoagulant series are evaluated with reference

to citrate as standard. A few values in each group have again been rejected as outliers. The standard deviations for citrate + heparin and citrate + sequestrene are again small, whilst the standard deviation for sequestrene is much greater. The order of probability for differences from citrate for each of the three other anticoagulant series is shown in Table IV. However, estimates of frequency are only tentative.

TABLE IV
PROBABILITY FOR DIFFERENCES FROM CITRATE

Difference from Citrate at One-hour Readings (mm.)	Sequestrene (%)	Sequestrene + Citrate (%)	Heparin + Citrate (%)
Greater than 10 ..	43·7	0·8	11·0
" " 15 ..	21·4	0·0	1·0
" " 20 ..	7·2	0·0	0·0
" " 25 ..	2·3		
" " 30 ..	0·4		

Assuming a normal distribution of differences between citrate (mean of three) and each of the other series, this table indicates the order of probability for differences (plus or minus) from citrate, taking into account the mean differences derived from the 20 cases.

Discussion

Dilution of heparinized and sequestrenized samples with citrate instead of physiological saline was chosen in order to obtain a quantitative comparison with the Westergren method, in which the same conditions of dilution and cell shrinkage apply. The added effects of heparin and sequestrene at their normal concentrations would, it was anticipated, have little effect in this respect.

It has been shown by Lewi and Clarke (1954) that dilution, far from being undesirable, tends to give a more accurate correlation of the sedimentation rate with clinical condition. In cases where the "rouleaux formation intensity" (R.F.I.), or sedimenting power, and packed cell volume are high, and similarly in cases where the R.F.I. is high and the P.C.V. is low, dilution increases the sedimentation rate. In cases where the R.F.I. and P.C.V. are normal (the normal range) the sedimentation rate is decreased. Tube bore also becomes important at increased plasma viscosity and increased P.C.V. (Shannon and Bywaters, 1957); however, the overriding factor is that of dilution. Using the Westergren method, one case was seen recently of primary atypical pneumonia with a P.C.V. of 56 ml./100 ml. and an E.S.R. of 5 mm. at one hour. On correction of the P.C.V. to 46 ml./100 ml. the E.S.R. was 80 mm. at one hour. It is evident that in this case the dilution employed in the standard method was still insufficient to bring the E.S.R. into the pathological range.

The results obtained in the present study show that dilution of heparinized or sequestrenized blood with 3.8% sodium citrate solution reduces the standard deviation between replicate readings to within the limits obtained for the standard Westergren method employing citrate alone. A similar finding has been demonstrated for dilution of oxalated blood with sodium citrate (Eastham *et al.*, 1958). Also, if the arithmetic mean of the 60 minute citrate reading is accepted as being the standard sedimentation rate for each blood sample, the average arithmetic means of the 20 readings for the sequestrene + citrate and heparin + citrate series differ by only +2 mm. and +4.5 mm. respectively from the average arithmetic mean of the 20 citrate readings. Since each sample has been separately diluted with sodium citrate for each of these anticoagulant mixtures, the scatter about the mean is consistent with sampling, diluting, and mixing errors.

It is therefore suggested that the Westergren sedimentation rate may be determined using blood which has been collected into Wintrobe oxalate mixture, heparin or sequestrene, simply by diluting four volumes of blood with one volume of 3.8% sodium citrate.

Summary

(1) The anticoagulant sequestrene is unreliable for determining the erythrocyte sedimentation rate. This has previously been demonstrated in the case of heparin and potassium/ammonium oxalate mixture.

(2) Blood which has been collected into oxalate, heparin, or sequestrene, when subsequently diluted with one-fourth of its volume of 3.8% sodium citrate, is suitable for determining the Westergren sedimentation rate.

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PLATELET SEROTONIN AND NORMAL HAEMOSTASIS

BY

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Serotonin, or 5-hydroxytryptamine (Rapport, 1949), the physiological function of which has not yet been established, is a powerful vasoconstrictor. Carried in the blood by the platelets (Humphrey and Jaques, 1954; Udenfriend and Weissbach, 1954) and released from them as clotting occurs (Zucker and Borrelli, 1955), it has frequently been suggested (Stewart and Zucker, 1913; Reid, 1943; Stefanini and Dameshek, 1955) that it may be concerned in the mechanism of haemostasis.

Recently it has been shown, however, that the platelets (and indeed the other body depots) can be deprived of their 5-hydroxytryptamine (5HT) by the action of the active alkaloids of *Rauwolfia serpentina*. The platelets not only lose the 5HT they carry but also their ability to take up any further 5HT, either *in vivo* or *in vitro* (Hardisty, Ingram and Stacey, 1956; Carlsson, Shore, and Brodie, 1957), yet no overt haemorrhagic tendency is met with clinically. Also Weiner and Udenfriend (1957) and Haverback, Dutcher, Shore, Tomich, Terry, and Brodie (1957) have studied, in considerable detail, the haemostatic mechanism in patients receiving reserpine and have failed to demonstrate any abnormality. This finding is of such fundamental importance that a further survey extending these observations seems desirable, and this we now present.

Methods and Materials

Twelve manic-depressive patients were studied. Control observations were first made upon six before treatment with reserpine began. Thereafter, all received for varying periods (none less than 14 days) a daily oral dose of 1.5 mg. "serpasil" (Ciba).

Standard Investigations.—These included: bleeding time (Duke's method), silicone clotting time (Biggs and Macfarlane, 1957), Hess's test, platelet count, prothrombin time (Quick's one-stage), prothrombin consumption index, and thromboplastin generation test (Biggs and Macfarlane, 1957).

The following additional observations were made:

Platelet 5HT Content.—This was assayed on rat colon treated with atropine after the method of Dalgliesh, Toh, and Work (1953), the extracts being made according to the method of Hardisty and Stacey (1955). The 5HT content is expressed as μg. free base/10^9 platelets.

Plasma Clot Retraction.—The platelet count of citrated platelet-rich plasma was adjusted to approximately 250,000 per c.mm. by the addition of platelet-free plasma. Five millilitres of this standardized plasma were placed in a siliconed graduated centrifuge tube and a weighted thread lowered into it. One hundred units of thrombin topical (Parke Davis), as 0.1 ml. of a 1,000 unit/ml. solution, were then added and, after being tilted to mix, the mixture was incubated for one hour at 37° C. The threaded clot was then withdrawn and its volume determined by reading the volume of the residual serum. With normally functioning platelets (unless the plasma fibrinogen content is abnormally high) the clot volume is 10% or less of the original plasma volume. This method of measuring the retraction was preferred as it eliminates the effect of variation in the packed cell volume.

Capillary Response to Injury.—This was measured by a method after Macfarlane (1941). Under the dissecting microscope, single capillaries of the nailbed were pierced by a short fine tungsten wire mounted in a glass rod and the response noted. With practice it was found possible to do this without the aid of a micro-manipulator. The glass fibres used by the original author were found to be difficult and time consuming to prepare. A fibre fine enough for use lacked the rigidity to penetrate the skin and more time was spent preparing glass fibres than observing the reactions of the capillaries. A $\frac{5}{1,000}$ in. or $\frac{10}{1,000}$ in. (125 μ and 250 μ approximately) tungsten wire can be brought to the required degree of fineness and sharpness simply by heating in a bunsen flame.* The shaft of the needle obtained from $\frac{2}{1,000}$ in. wire was found to be insufficiently strong. The skin was cleared with cedarwood oil and the best illumination was obtained from a highly intense source of green

*We are indebted to Mr. J. T. Lloyd, Department of Natural Philosophy, Glasgow University, for this method of preparing fine wires.

light. The examination is carried out with success only in fingers where the cuticle has been allowed to grow naturally up the nail.

Platelet Adhesiveness.—Three millilitres of platelet-rich plasma, which had been collected with a siliconed system and kept at 4° C. during all manipulations, were placed in a 15 ml. siliconed centrifuge tube with a silicone rubber stopper, the tube revolving on a "matburn"* mixer at 33 revolutions per minute in a refrigerator at 4° C. The time taken for the platelet count to drop to half its initial figure was taken as a measure of the stickiness, the counts being performed at 30-minute intervals. The normal range of "half-time" by this method is one and a half to three and a half hours.

Thromboplastin Generation Test with Platelet Dilutions. — The thromboplastin generation test, having initially been carried out with a platelet concentration of 1×10^6/c.mm., was repeated with serial 1 in 2 dilutions of the platelet suspension. All other components of the system were standardized over the whole series of tests and controls, the only variables being the different subjects' platelets in their several dilutions. It was, therefore, appropriate to compare the performance in the generation of thromboplastin of the platelets from different donors. In a series of 21 normals, no effect on thromboplastin generation was found until the platelet suspension was diluted to 1 in 8 or more. In three cases this dilution test was also carried out with platelet suspensions to which had been added synthetic serotonin in a strength of 0.5 μg./10^9 platelets.

Results

Hess's test, prothrombin times, platelet adhesiveness, and clot retraction showed no change from normal after the treatment with reserpine.

Capillary Response to Injury.—The responses in the patients given reserpine as in the controls were normal, i.e., in all patients capillaries were found which vanished for 20 minutes or more after injury. As Macfarlane (1941) described, occasional capillaries were also noted which disappeared only momentarily or were only partially obliterated by irregular constrictions. Bleeding for more than the usual four or five seconds, which is of diagnostic significance in some forms of non-thrombocytopenic purpura, did not take place.

Thromboplastin Generation Tests.—No change was found in the generation of thromboplastin after the giving of reserpine. Dilution of the platelet suspension only affected generation when 1 in 8 or more. The addition of synthetic serotonin to the platelet suspension in no way affected generation.

The other findings are listed in the Table.

*Matburn Ltd., 25, Red Lion Street, London, W.C.1.

TABLE

Case	Platelets (10^9/c.mm.)	Bleeding Time (min.)	Silicone Clotting Time (min.)	Prothrombin Consumption Index	5HT (μg. Free Base/10^9)
I (a)	355	2	21	5	0·59
(b)	320	1½	22	8	Nil
II (a)	320	2½	22	3	0·51
(b)	280	2½	22	7	Nil
III (a)	320	2½	24	10	0·71
(b)	340	2	22	9	Nil
IV (a)	290	3	18	3	0·45
(b)	280	3	18	14	Nil
V (a)	210	2	20	2	0·54
(b)	165	2	22	10	Nil
VI (a)	335	2	24	3	0·39
(b)	320	2	24	9	Nil
VII (b)	305	2½	23	13	,,
VIII (b)	280	3	21	2	,,
IX (b)	230	3	22	25	,,
X (b)	330	3	19	4	,,
XI (b)	225	2	19	11	,,
XII (b)	310	2	19½	2	,,

(a) = Pre-treatment. (b) = Post-treatment.

Discussion

The fact that 5HT is released from platelets during clotting suggests that it may take part in haemostasis normally. If so, a lack of 5HT might be expected to reduce the efficiency of the mechanism and an excess perhaps to enhance it.

While a transitory excess of serotonin, produced by intravenous administration, has apparently resulted in a decreased bleeding time in certain animals (Correll, Lyth, Long, and Vanderpoel, 1952), this is as likely to have been due to generalized vasoconstrictor action as to a purely local one at the site of injury. Only an evanescent and much less striking reduction in the bleeding time has been described in man after intravenous serotonin (Stefanini and Magalini, 1956) and no such findings have been recorded in cases of carcinoidosis.

Lack of 5HT, on the other hand, has not prolonged the bleeding time at all in the patients given reserpine investigated either by ourselves or others and this surprising finding seems to exclude 5HT from being concerned in the vasoconstriction of haemostasis. Estimation of the bleeding time involves injury both to capillaries and to larger vessels with muscular walls. According to Reid (1943) human capillaries are not susceptible to the action of the serum vasoconstrictor, and our direct observations show that capillary constriction following trauma can occur in the absence of 5HT. Muscular-walled vessels, however, do respond to the action of the serum vasoconstrictor (Reid, 1943; Zucker, 1947). Lack of 5HT might therefore have been expected to lessen their response and so prolong the bleeding time. Against this Zucker (1947) has shown that trauma

alone will cause such vessels to contract. She noted that contraction followed injury of muscular-walled vessels in rat mesentery, even in thrombocytopenic animals where no platelet plug formed and where, therefore, there could have been no release of 5HT. From all these findings it seems that release of 5HT is not essential for the effective contraction of an injured vessel.

Although evidence is lacking that 5HT is related to physiological haemostasis by virtue of its vasoconstrictor activity, platelets have other functions in haemostasis and it has to be considered whether these might be altered by the absence of 5HT brought about by reserpine. Fenichel and Seegers (1955) have noted that clots of diluted platelet-poor bovine plasma retracted when serotonin was added in adequate concentration, but we found no diminution in clot retraction in the standardized platelet-rich plasma when the platelets had been freed of their 5HT; nor was intrinsic platelet adhesiveness altered. Although reserpine so intimately affects the platelets as to release all their 5HT this had no demonstrable effect on their contribution to thromboplastin generation either, their activity being undiminished even at a considerable dilution. Furthermore the normal prothrombin consumption indices indicate that the second stage of coagulation is not materially affected by the lack of 5HT. Lastly, it has been shown (Correll *et al.*, 1952) that the presence of 5HT does not accelerate the third stage of coagulation, namely, the conversion of fibrinogen into fibrin. Our conclusion is that the removal of 5HT from the blood of a normal person causes no haemostatic defect, and this finding is in keeping with clinical observations and experience in disorders of the blood (Hardisty and Stacey, 1957). Recently, however, Jaques, Fisher, and Ashwin (1958) have reported that the superimposition of stress (10% NaCl intraperitoneally) on rabbits given reserpine or of adrenalectomy in rats given reserpine provoked a haemorrhagic death; the significance of this finding is not clear.

Summary

Twelve patients whose platelets had been freed of 5-hydroxytryptamine by the administration of reserpine showed no demonstrable defect in their haemostatic mechanism. From this it appears that 5-hydroxytryptamine plays no part in normal haemostasis.

We are indebted to Dr. Angus McNiven for his permission to carry out these investigations on patients under his care; to Professor D. F. Cappell for helpful criticism and advice in the preparation of this paper; to Mr. L. Nicolson, F.I.M.L.T., and Miss P. Cassidy, B.Sc., for technical assistance.

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THE PRACTICAL VALUE OF THE FROG TEST IN THE DIAGNOSIS OF NORMAL AND PATHOLOGICAL PREGNANCY

BY

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The laboratory diagnosis of pregnancy, initiated by the discovery of the Aschheim-Zondek reaction, remained for a long time a comparatively seldom used procedure, performed only on selected cases. The original method and its later modifications (the Friedman test, the quick hyperaemia test) need comparatively expensive test animals. Moreover, the performance of the test and reading of the results is by no means always easy and requires a highly skilled laboratory staff. One of the simpler procedures, the female Xenopus Lewis frog test, although much advocated for some time, was found to lack sensitivity and the animals were difficult to breed and maintain outside their native land. The various chemical procedures proposed lacked both sensitivity and specificity and therefore have been disappointing. A new era in this field was initiated by Galli-Mainini, who described the use of several species of South American male frogs for the diagnosis of pregnancy (Galli-Mainini, 1948). The procedure became universally known as the "frog test" and very favourable reports about its use were soon published in the medical press of many countries (Wiltberger and Miller, 1948; Haines, 1948; Hinglais and Hinglais, 1948; Nobili, 1948; Rabau and Szejnberg, 1949). The following is a summary of experience with this procedure during nearly 10 years of its routine use.

In nature the male frogs and toads release spermatozoa after a prolonged contact with the female, which stimulates the secretion of the gonadotrophic hormone from the hypophysis. A similar effect may be obtained by injecting human chorionic gonadotrophin excreted in urine during pregnancy, or in cases of hormone-secreting neoplasms (chorionic epithelioma, hydatid mole).

Test Animals

The suitability of the *Batrachia* for this procedure seems to be universal. Bhaduri in 1951 listed from the literature 34 species of frogs and toads belonging to 11 genera and eight families responsive to the injection of human chorionic gonadotrophin (Bhaduri, 1951). The three species indigenous to Israel (belonging to three different families, namely, the toad *Bufo viridis*, the river frog *Rana ridibunda*, and the tree frog *Hyla arborea*) were found suitable for the reaction (Szejnberg and Rabau, 1950). The care of the animals is exceedingly simple. In large laboratories where hundreds of frogs are used they may be kept in big tanks or aquaria with little water. In small laboratories or in a doctor's surgery any small glass jar placed in the shade may be used. The only care required is the daily change of water. Occasionally a fatal epidemic of the so-called "red leg disease" may spread in aquaria, but it may be overcome by sprinkling the aquaria with sulphadiazine or adding penicillin-streptomycin solution to the water, and promptly removing dead animals. In small laboratories where each test animal is kept in a separate container no danger of an epidemic exists.

The animals in captivity do not show much inclination for food (live worms) and sometimes have to be forced to swallow it. On the other hand, they seem to withstand very well prolonged periods of hunger extending even to several months. Although they become considerably emaciated during this period, they regain weight very easily when ultimately released. The usual procedure here is to use the animals for two or three months without feeding and then to release them.

The animals may be used repeatedly at an interval of at least seven days between consecutive injections, provided a control examination of the animals' urine is always made before the injection. On a few occasions spermatozoa were found in the urine of animals even a week after a previous positive response. Although the number of such cases was very small, they warranted a careful examination of each animal before use.

Technique

The urine (1 ml./10 g. weight of animal) is injected subcutaneously into the dorsal lymph sac of the test animal. Before the injection and two hours after it a specimen of the animal's urine is procured from the cloaca with a capillary pipette and observed under the

microscope. If negative, the examination is repeated after four hours.

Occasionally the urine is toxic to the animals as a result of drugs taken by the patients or to some unknown cause. This difficulty may be overcome in two ways: (1) By injecting a smaller volume of urine. This may be sufficient to give a positive result. If, however, the test is negative with the smaller volume, then the test should be repeated with the full amount of detoxified urine. (2) Of the various methods for detoxification, the following was found to be the most suitable for routine use: to 5 ml. of urine 20 ml. of ice-cold acetone is added, mixed, and left in the refrigerator for two hours. After centrifuging the supernatant is discarded, the precipitate washed once with cold acetone, and centrifuged again. The washed precipitate is left in the tube for 30 min. at room temperature in order to evaporate the traces of acetone, and resuspended in 5 ml. water. Alternative efficient methods of detoxification which are quicker, requiring only 30 to 60 minutes for completion, but are technically somewhat more complicated were described by Scott (Scott, 1940; Brody, 1949; Cutler, 1949) (absorption from acidified urine with kaolin and elution with alkali) and by Robbins (dialysis of urine against running tap water) (Robbins, 1951).

Sensitivity of the Animals

The sensitivity of the animals was tested by injection of saline dilutions of international standard human chorionic gonadotrophin and calculating the ED50. A considerable seasonal variation in the sensitivity of the animals was observed. The *Bufo viridis* was about twice as sensitive during the winter season (time of growth and reproductive activity) than in the summer months. In winter 3 international human chorionic gonadotrophin units were sufficient to cause a positive response in 50% of the injected animals, while in summer about 6 units were required to cause 50% positive responses. The sensitivity changes in *Rana ridibunda* differed. This animal was more sensitive in summer (ED50 2.5 international units) than in winter (3.5 international units). The tree frogs (*Hyla arborea*) were used only during the winter and spring seasons, as they did not survive the summer in captivity. These small frogs weighed on the average 4–5 g. and their sensitivity range was about 0.6 to 0.8 international units human chorionic gonadotrophin.

The sensitivity of the animals did not change significantly after being used several times. Taking into account the individual and seasonal variations of sensitivity, no test was reported negative unless three animals were injected (preferably belonging to the three different species) and found negative.

Quantitative Procedure

For the quantitative estimation of the hormonal content two-fold dilutions of urine in water were prepared and 1 ml. of each dilution injected into the test animals. The maximal dilution giving a positive response was noted and the amount of the hormone in urine calculated as follows:

Dilution of urine × 1,000 (frog units/litre).

Frog units/litre × mean sensitivity of the test animals (different for different seasons and animals) human chorionic gonadotrophin international units/litre.

Results

Diagnosis of Pregnancy. — About 7,000 pregnancy tests have been performed during the period covered by this summary. The accuracy of the test depended on the length of amenorrhoea. The earliest examinations were performed four days after the date of the missed period. In some cases positive results have been obtained at such an early date, but on many occasions the test was negative to become positive a week later. The incidence of the positive results rose with each consecutive day of amenorrhoea, and with urines examined 12 days after the missed period or later no false negative results were found in our experience.

No false positive results were encountered when the test was performed with *Bufo viridis* or *Hyla arborea*. *Rana ridibunda*, on the other hand, was less reliable. On very rare occasions during the summer months only, a few spermatozoa could be detected in cases which later proved to be negative. These false positive results were probably caused by unspecific release of a few spermatozoa, as four or five additional *Rana* frogs injected with the same urine gave a uniformly negative result. Consequently, when during the summer months a very weak positive reaction was encountered in *Rana ridibunda* and additional animals injected were negative, the test was reported as inconclusive and an additional specimen of urine was requested for examination seven days later. Such cases were very rare, and with the above precautions no false positive results were reported by us during the entire period.

Value of Quantitative Test in Pathological Pregnancies.—The quantitative test has been used for the diagnosis of missed abortions as well as in the follow up of cases of habitual abortion. As a preliminary step for these investigations excretion levels of chorionic gonadotrophin in 200 normal pregnancies have been followed. It was found that individual variations in the range of excretion were very wide, but by calculation of the mean values for each gestation period, a hypothetical "normal curve" of chorionic gonadotrophin excretion could be drawn. This "normal curve" is characterized by a peak excretion during the 60–110th day of amenorrhoea.

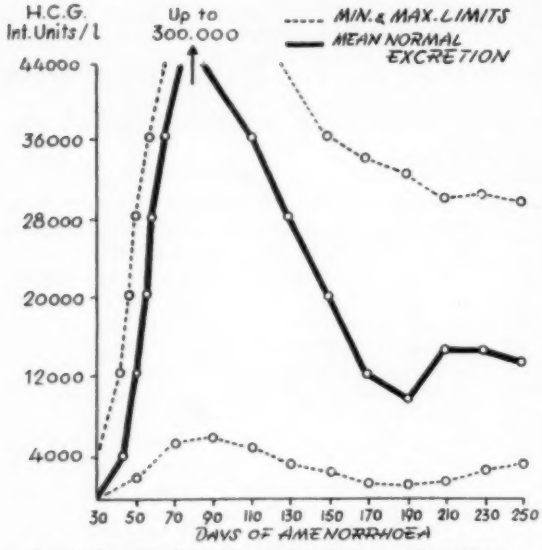

FIG. 1.—Excretion of chorionic gonadotrophin in normal pregnancies.

During this peak period most of the subjects excrete more than 32,000 international units chorionic gonadotrophin. After this period the levels usually drop, and during the fifth to sixth month of pregnancy very low values are encountered, approaching in some cases the limit of sensitivity of the test animals. Later the "mean value" rises again although to much lower levels than during the peak period (Fig. 1).

The second preliminary step consisted of investigating the rate of disappearance of chorionic gonadotrophin from urine after interruption of pregnancy. For this purpose the levels of the hormone were estimated before and after curettage. The results presented in Fig. 2 show that the speed of disappearance of the hormone from urine was related to the initial level before the intervention. When it was low, the urine had already become negative two or three days after the interruption of the pregnancy, while in cases with high initial levels even 14 days were sometimes required for its decrease below the sensitivity level of the test animals (Fig. 2).

Up to now about 500 cases of suspected missed abortion have been investigated by the quantitative method, and the following conclusions can be drawn.

(1) A single estimation of the gonadotrophin level cannot serve as a basis for the diagnosis of foetal death unless the frog test is negative. When the examination was performed a few days after the suspected foetal death the hormonal level could be still very high (especially during the "peak period" of the hormonal excretion). On the other hand, the hormone titre could be low in cases when the foetus was still alive and normally developing, particularly during the fifth to sixth month of pregnancy, but also in some cases during the first trimester.

(2) Only repeated quantitative examinations performed at about one week's intervals can be of diagnostic value in cases of missed abortions. If a rise in the hormonal level or even maintenance of a "plateau" is detected on two consecutive examinations, the diagnosis of foetal death may be discarded with certainty. When, on the other hand, a decline in the level is observed after uterine bleeding or other unfavourable clinical signs (disproportion between the gestation period and the size of the uterus) a correlation with the

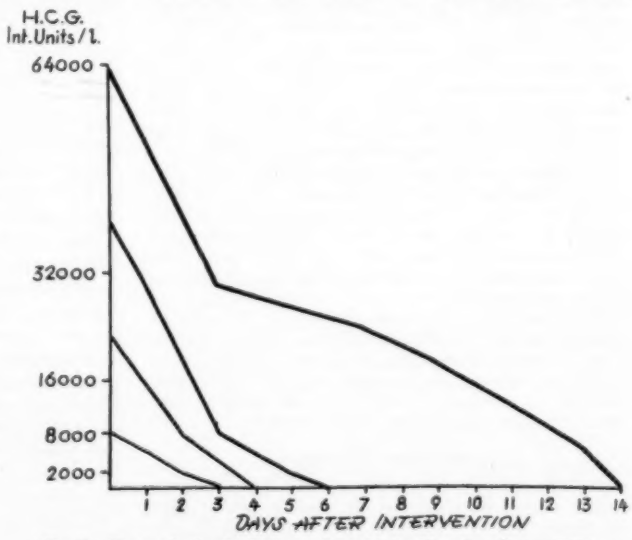

FIG. 2.—Changes in the chorionic gonadotrophin level in urine after curettage.

normal trend of the "mean normal excretion curve" should be made and the case supervised carefully by repeated examinations until a final conclusion can be reached.

Follow-up in Cases of Habitual Abortion.—The quantitative test has been performed at fortnightly intervals in about 100 cases of habitual abortion (cases with at least two previous spontaneous abortions).

(1) The chorionic gonadotrophin level in most cases of habitual abortion was within normal limits, the mean levels not being significantly lower than the mean normal values.

(2) The quantitation of the hormone had no prognostic value for the normal continuation of pregnancy. In some cases a finding of high hormonal excretion was followed a few days later by foetal death, while in other cases the pregnancy terminated well despite prolonged very low levels of the hormone and initial bleeding.

(3) While of no prognostic value, the test was very useful for the diagnosis of the foetal condition when performed regularly at two-week intervals. In most cases it eliminated the necessity for repeated gynaecological examinations, which in cases of habitual abortion were usually not easily agreed to by the patients and caused them unnecessary psychological stress. These patients often blame the doctor if foetal death occurs a short time after the physical examination. By these repeated tests foetal death can be relatively quickly diagnosed and the dead foetus removed, thus preventing unnecessary confinement to bed and the danger of fatal haemorrhage due to fibrinolysis.

(4) Some of the patients with a history of habitual abortion were given stilboestrol or progestine, but no medication was prescribed to the other patients. These drugs did not appear to affect the level of chorionic gonadotrophin excretion even when doses up to 100 mg. daily were given.

Summary

The "frog test" described by Galli-Mainini is a very useful procedure for the diagnosis of pregnancy. It is characterized by technical simplicity and a very high degree of accuracy.

Periodical hormonal estimations by this procedure are recommended during pregnancy in cases of habitual abortion and in cases of suspected missed abortions for prevention of unnecessary confinement to bed and of dangerous haemorrhagic complications due to fibrinolysis.

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PULMONARY HAEMORRHAGE WITH EXTENSIVE GLOMERULAR DISEASE OF THE KIDNEY

BY

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Under the title "Haemorrhagic and Interstitial Pneumonia with Nephritis," Parkin, Rusted, Burchell, and Edwards (1955) described seven cases in which gross haemorrhage into the alveoli of the lungs occurred in association with a severe glomerular lesion of the kidneys. The present paper describes three cases seen at St. Mary's Hospital during the past four years in which a similar association was found, and an attempt is made to consider the nature of such an association.

Case Histories

Case 1.—This patient, a housewife, was first seen in 1948, then aged 61 years, because of osteoarthritis of the knees. She was not seen again till 1956 when she attended hospital because of vomiting, nausea, and abdominal pain of one year's duration. She had been short of breath for five weeks. A diagnosis of hiatus hernia, confirmed by barium meal, was made, the haemoglobin at this time being 13.3 g./100 ml. During the next seven weeks she became weaker and vomiting accompanied by abdominal pain became a daily occurrence. Her relatives remarked that she had been having dark stools for four weeks. She showed a marked personality change, being now depressed instead of cheerful. There was no relevant family history.

On admission to hospital she was dyspnoeic and pale with no evidence of cyanosis. Arthritic changes were present in both knees. There was a moderate blowing systolic murmur over the whole heart, especially the apex, the heart rate being 100/minute and regular. There was no finger clubbing and no fever; blood pressure 165/95 mm. Hg; Hb 8.1 g./100 ml.; R.B.C. 3.0 × 10^6/c.mm.; P.C.V.=30, M.C.V.=100 $c\mu$, M.C.H.=27 $\gamma\gamma$, M.C.H.C. 27%. The urine contained much protein, moderate numbers of red blood cells, and a few casts. A specimen of stool was weakly positive for occult blood. Investigations carried out during the next few days were: blood urea 370 mg./100 ml.; plasma protein 5.3 g./100 ml. (albumin 2.2 g., globulin 3.1 g.); serum cholesterol 100 mg./100 ml.; serum electrolytes (mEq./litre) Na 135, K 7.7, CO_2 12.5, Cl 99. There was a transitory symptomatic improvement after intravenous dextrose and sodium lactate, but after a haemoptysis of a few ounces of blood on December 11 death occurred the following day.

Relevant Necropsy Findings.—Body weight was 68 kg. The heart weighed 395 g. with slight ventricular thickening and some tiny vegetations on the mitral valve. There was no stenosis of the mitral valve. The trachea and bronchi contained frothy blood. Externally the lungs, which were large and bulky, showed many purple areas. On section there was much fluid blood throughout the lung with some firmer granular dark areas. There was no obvious source of haemorrhage. The subcapsular surfaces of the kidneys (right 168 g., left 125 g.), which were smooth, showed petechiae. On section there was red and white cortical mottling, the rest being pale with a few medullary petechiae.

Microscopic Changes. — The most conspicuous change was the presence in alveoli of large numbers of red blood cells with or without fibrin. The change was diffuse except that small scattered areas showed very few red blood cells. Some of the fibrin showed evidence of organization and a certain number of alveoli showed watery fluid. Siderophages were present in alveoli but were not so numerous as in the following case. Interalveolar septa showed only scattered abnormalities consisting of fibrous thickening or proliferation of the lining cells. The reticulin and elastic distribution in the septa was not materially abnormal and no septal necroses were found. There were no changes in bronchial or pulmonary arteries and there was no evidence of infection.

Almost all glomeruli were affected, although to different degrees. Fibrinoid necrosis of the tufts was a frequent finding (Fig. 1), affecting usually only a part of the tuft but sometimes affecting the whole. In the latter instances the necrosis was continuous with a similar change in the afferent arteriole. Proliferation of endothelial and epithelial cells was frequent in the tufts, and localized areas of hyalinization or fibrosis were present in some. Some appeared to be completely sclerotic. There was considerable proliferation of cells lining Bowman's capsule and well-developed crescents, some of which showed organization, were conspicuous.

There was much loss of proximal convoluted tubules whilst others showed dilatation. Red blood cells and casts were frequent, whilst some tubules showed iron pigment.

Interlobular arteries showed the changes of polyarteritis nodosa in an acute stage with fibrinoid

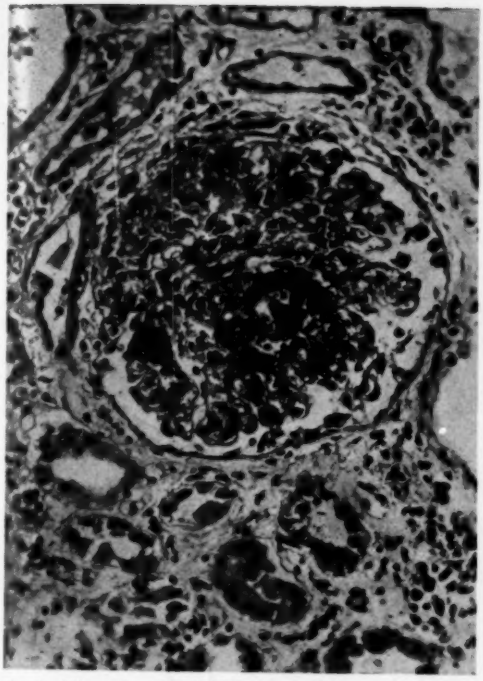

FIG. 1.—Glomerular tuft with fibrinoid necrosis in Case 1. Haematoxylin and eosin, × 240.

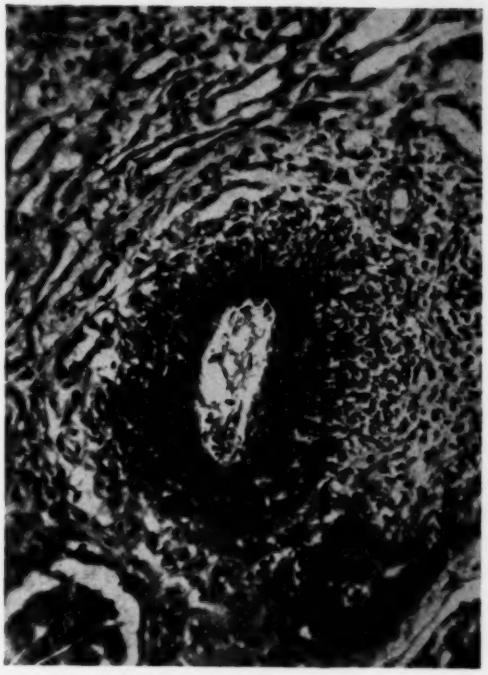

FIG. 2.—Interlobular artery with fibrinoid necrosis and cellular reaction in Case 1. Haematoxylin and eosin, × 170.

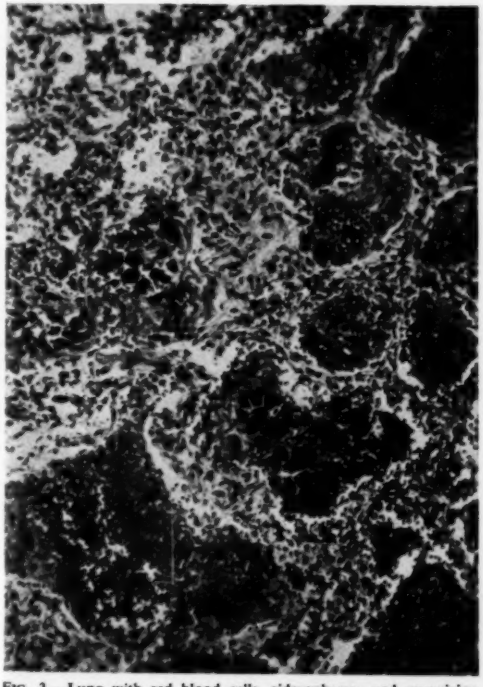

FIG. 3.—Lung with red blood cells, siderophages, and organizing fibrin in alveoli in Case 2. Haematoxylin and eosin, × 140.

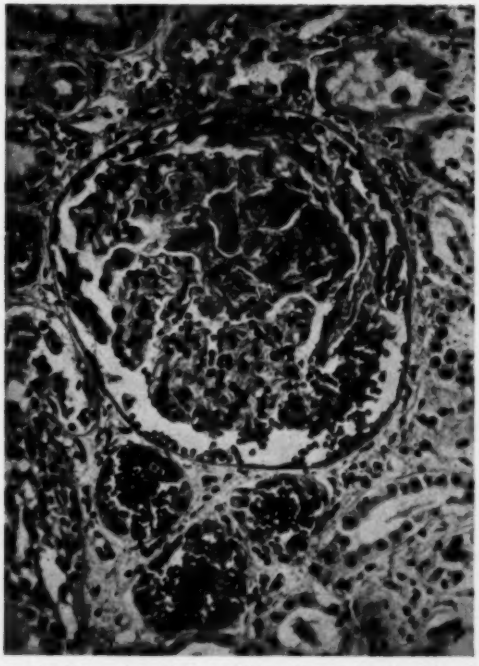

FIG. 4.—Glomerulus with focus of fibrinoid necrosis and red blood cells in Bowman's space in Case 2. Haematoxylin and eosin, × 240.

necrosis and cellular reaction (Fig. 2). Other vessels of this size showed much fine intimal collagenous thickening. Some afferent arterioles showed fibrinoid necrosis continuous with necrosed tufts. Several arcuate arteries showed fibro-elastic intimal thickening.

In the interstitium there were large numbers of plasma cells and a fine fibrosis. The arteries of the spleen showed acute polyarteritis nodosa.

Case 2.—This patient, a man aged 23 years, felt off colour and short of breath in mid-July, 1954. He developed a slight cough and brought up fresh blood on several occasions. The greatest amount of blood was 1 oz. but usually it was merely streaking in the sputum. At the beginning of September he complained of indigestion and vomited on one occasion. His breathlessness increased. In the middle of September he was found to have moderate proteinuria, and white and red blood cells and granular casts in the urine, which was sterile. Other investigations showed: Hb 8 g./100 ml.; R.B.C.s 2.01m./c.mm.; W.B.C. 11,800/c.mm. (80% polymorphs), platelets 80,000/c.mm.; blood urea 90 mg./100 ml.; serum protein 5.83 g./100 ml. (albumin 4.93 g., globulin 0.9 g.); indirect serum bilirubin 0.4 mg./100 ml. A transfusion of packed red cells was attempted, but a shivering attack caused this to be abandoned.

He was admitted to St. Mary's Hospital on September 27, 1954, in a desperately ill condition. There was no relevant family history and nothing important in the past history except that as a boy he used to get unexplained pyrexia and a vague history of nocturnal frequency of micturition. He was pale, slightly pyrexial, but not oedematous. The pulse was regular, there were no cardiac murmurs, and blood pressure was 130/70 mm. Hg. Chest radiographs showed increased shadowing obscuring the mid-zones peripherally and extending into the upper zones which showed coarse miliary mottling. Investigations carried out were: Hb 3.7 g./100 ml.; R.B.C. 1.55m./c.mm.; W.B.C. 11,000/c.mm. (85% polymorphs); platelets 155,000/c.mm.; blood urea 184 mg./100 ml.; serum protein 5.6 g./100 ml. (albumin 3.75 g., globulin 1.85 g.). The urine contained a large amount of protein, abundant red blood cells, a moderate number of pus cells, and granular casts in the deposit. He was treated with intravenous sodium bicarbonate, blood transfusions, and, because of the possibility of miliary tuberculosis, with streptomycin and isoniazid. The blood urea continued to rise and two days later was 220 mg./100 ml. He coughed up large amounts of blood-stained sputum and died four days after admission.

Relevant Necropsy Findings (Dr. R. R. Wilson).—Body weight was 62 kg. The heart weighed 456 g. with some left ventricular thickening but no valvular defects. The lungs were dark and solid throughout. In addition there were numerous small petechial haemorrhages under the pleura. There was blood-stained fluid in the bronchi, but no obvious cause for the haemorrhage. Both kidneys (right 196 g., left 228 g.) were swollen, soft, and congested with petechial haemorrhages under the capsule and on the cut surface.

Apart from some petechial haemorrhages in the white matter of the brain, there were no other remarkable changes.

Microscopic Changes.—The main change in the lung consisted of large numbers of red blood cells with small numbers of polymorphs in the alveoli, sometimes associated with fibrin which in places showed organization by fibroblasts. There were also large numbers of haemosiderin-containing macrophages (siderophages) in alveoli, sometimes being found in organizing fibrin (Fig. 3). These changes were diffuse throughout the lung. In some alveoli there was oedema fluid, although this was not conspicuous, and occasional hyaline membranes were seen. Inter-alveolar septa usually showed no change, but some showed dilated capillaries and others focal fibrous thickening. The reticulin pattern of the septa was preserved and the elastica of both septa and blood vessels showed no remarkable change. The septa showed no striking increase in cells and there were no recognizable necroses. Occasionally siderophages were incorporated into the septa. Pulmonary and bronchial arteries were normal. Apart from containing red blood cells, bronchi and bronchioles were normal.

All the glomeruli were affected and showed changes of approximately the same age. The tufts were reduced in size and showed an increase in hyaline material and fine collagen. There was some cellular proliferation in the tufts, and scattered glomeruli showed intracapillary thrombosis which in places was continuous with the capillary wall and resembled fibrinoid necrosis (Fig. 4). There was generalized proliferation of the epithelium lining Bowman's capsule, producing epithelial crescents encroaching considerably on the capsular space (Fig. 5). There was no organization of crescents. Red blood cells were frequent in the capsular space.

Some proximal tubules were lined by a low type of epithelium and large numbers of red blood cells were present in tubules of all types.

Apart from a thrombus in a large renal vein there were no vascular changes. In particular there were no changes of acute arteritis and no evidence of healed arteritis.

Interstitial tissue showed no changes.

Case 3.—A 41-year-old farmer noticed some malaise during the early part of 1957. In October of 1957 he was admitted to hospital in Ireland for six weeks because of shortness of breath on exertion and dyspnoea while lying flat in bed. At this time there was no cough, no chest pain, and no ankle oedema. He had a haemoptysis at this time and was told while in hospital that he had blood in the urine. During his stay in hospital he had pain in the left loin lasting for three days. Shortly after discharge he developed ankle oedema.

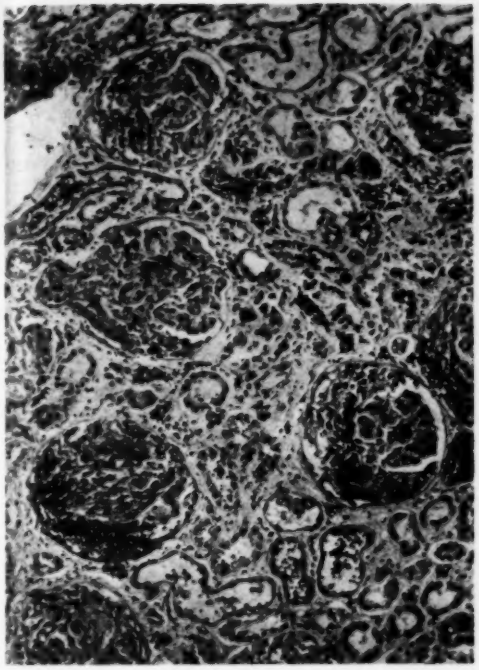

FIG. 5.—Glomeruli to show crescent formation in Case 2. Haematoxylin and eosin, × 100.

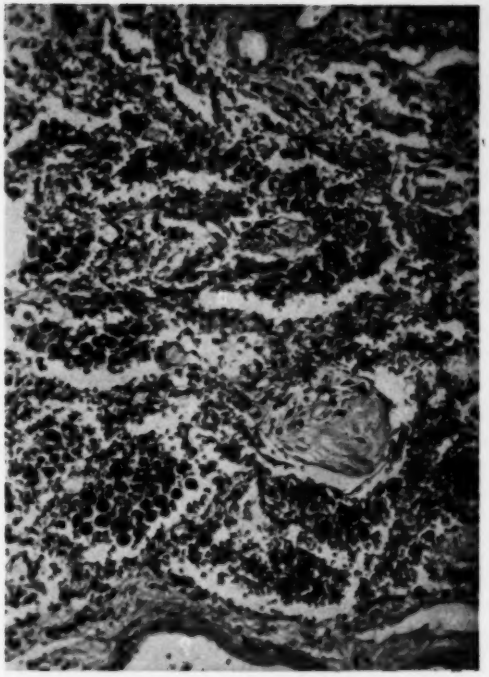

FIG. 6.—Lung with red blood cells and siderophages in alveoli in Case 3. Haematoxylin and eosin, × 140.

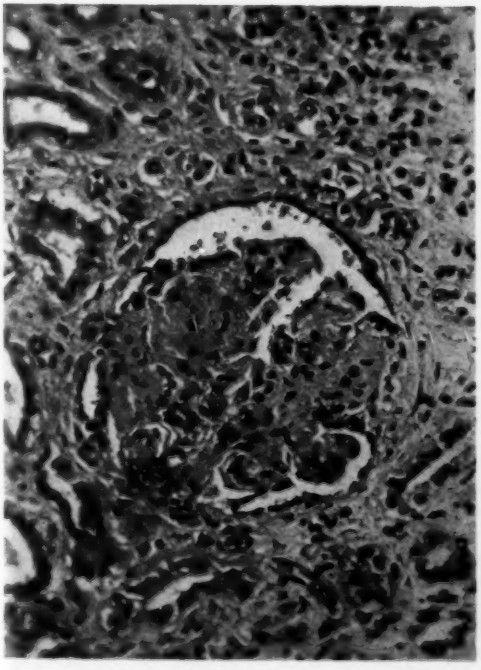

FIG. 7.—Glomerulus with hyalinization in Case 3. Haematoxylin and eosin, × 240.

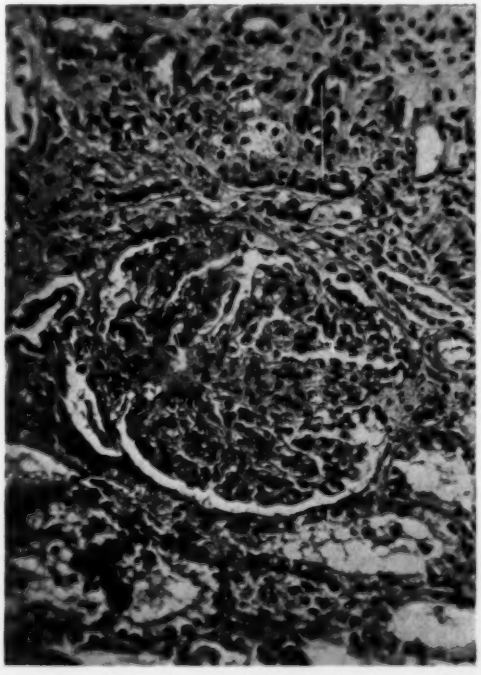

FIG. 8.—Glomerulus with cellular proliferation in Case 3. Haematoxylin and eosin × 170.

During the early part of January, 1958, he developed a productive cough with sputum containing a considerable quantity of bright red blood. He was admitted to St. Mary's Hospital on January 21, 1958.

He was a tall, muscular, cheerful man with no relevant family history except that his father, who died at 66 years, had suffered from asthma. Eight brothers and sisters were alive and well. There was no history of rheumatic fever, scarlet fever, chest disease, or nephritis. He had suffered from frequency of micturition, both day and night, since childhood, but this had not been worse recently. There was no recent loss of weight and appetite had been good up till three days before admission.

On examination he was moderately dyspnoeic at rest, there was no cyanosis and no clubbing. The pulse rate was 120 and regular; blood pressure 180/110 mm. Hg; the heart enlarged to the left with a blowing apical systolic murmur and a loud mid-diastolic sound. There was both ankle and sacral oedema and a jugular venous pressure of 4 cm. The ocular fundi were normal. Investigations performed: E.S.R. (Westergren) 80 mm. in one hour; serum cholesterol 208 mg./100 ml.; total serum protein 5.5 g./100 ml. with albumin 50% of total; blood urea 180 mg./100 ml.; Hb 6.7 g./100 ml. Urine: protein 3.2 g./24 hr., with 2.7×10^6 leucocytes and non-squamous epithelial cells per hour. A test for occult blood in the stool was negative.

During the first few days in hospital the patient produced between 3 and 5 oz. heavily blood-stained sputum daily. A chest radiograph showed considerable cardiac enlargement and generalized pulmonary oedema. He was given digitalis to control the cardiac failure and was transfused with 2 pints of packed red blood cells. There was a marked lessening of dyspnoea and signs of cardiac failure disappeared. Linear haemorrhages, however, developed in the retina and the blood pressure rose to 200/140 mm. Hg, at which time he was treated with mecamylamine and reserpine. Blood cultures were sterile and no organisms were grown from the urine.

In spite of a 3,000 calories low-protein diet and 3 litres of fluid daily he became increasingly acidotic and the blood urea continued to rise. Oral feeding was superseded by intravenous drip therapy.

On February 6, 1958, he was started on prednisone, 60 mg. daily, for two weeks, after which time the dosage was reduced to 30 mg. daily. Two days after the start of this therapy haemoptysis ceased and never recurred.

Following a six-hour dialysis on February 9 (Dr. Victor Wynn) the blood urea was reduced from 290 mg./100 ml. to 80 mg./100 ml., and for 11 days he was much improved. However, because of the fall in urinary output and the rising blood urea, a further dialysis was performed on February 20, but death occurred the following day.

Relevant Necropsy Findings.—Body weight was 78 kg. The heart weighed 580 g. There was a slight fibrinous pericarditis and hypertrophy of the left ventricle. No valvular defects were present. The lungs were heavy and bulky. The lower lobes showed subpleural purplish areas. On the cut surface there were numerous firm red-brown areas superimposed on a rather moist, moderately dark background. Blood was present in the trachea and bronchi. No obvious cause for haemorrhage was found. The right kidney weighed 135 g. and the left 118 g. The left kidney showed some blood outside the capsule (presumably from an unsuccessful needle biopsy of kidney some days before death). Both kidneys were reduced in size and showed thinning of the cortex with finely granular subcapsular surface.

Microscopic Changes.—The most striking changes were the large numbers of red blood cells and siderophages in alveoli (Fig. 6). Organizing fibrin was a frequent finding, siderophages often being embedded in the organizing mass. Inter-alveolar septa showed engorged capillaries and some showed fibrosis, often in continuity with organizing fibrin in the alveoli. The septal reticulin pattern was preserved except in scattered areas which showed patchy bronchopneumonia. The elastica in both septa and blood vessels showed little change. There was no increase in inflammatory cells in septa. There were no pronounced arterial changes.

The alveolar haemorrhages were more pronounced in the lower lobes than elsewhere.

Almost all glomeruli were affected, the degree of involvement varying considerably. Many were completely hyaline, reduced in size, and surrounded by a mantle of collagen. Others showed partial or complete hyalinization, the tuft being covered by proliferating epithelial cells (Fig. 7). Others showed proliferation of endothelial cells in the tufts (Fig. 8), but no fresh necroses were seen and there was no crescent formation.

There was considerable tubular loss, remaining proximal tubules being dilated and containing eosinophilic casts.

Arcuate arteries showed fibro-elastic intimal thickening, sometimes affecting only a part of the circumference. Interlobular arteries showed fine cellular collagenous thickening which was pronounced in most. There were hyaline changes in arterioles. No evidence of polyarteritis nodosa in the acute stage was seen.

There was fine interstitial fibrosis and scattered lymphocytes and plasma cells.

Discussion

From the clinical point of view the cases showed many similar features. All suffered from abdominal discomfort, dyspnoea, profound anaemia, haemoptysis, and evidence of renal failure. The only haemoptysis in Case 1 occurred the day before death, but in Cases 2 and 3 it was present for two and four months respectively. There was a history of nocturnal frequency of micturition in Case 3 and a vague history of

frequency in Case 2. The third patient was the only one with any appreciable rise of blood pressure.

At necropsy it was found that all showed enlargement of the heart, the left ventricles being hypertrophied in the absence of any gross valvular defects. There is a fundamental similarity of the renal lesion in Cases 1 and 2, while in Case 3 an older type of change is present. Case 1 represents the necrotizing glomerulitis so frequently associated with polyarteritis nodosa, evidence for the latter being provided by acute arterial lesions in the kidney and spleen. This particular type of renal change was well described by Davson, Ball, and Platt (1948), and the kidney changes in Case 1 conform well to their description. The spectrum of involvement of glomeruli is wide in this case, for at one extreme some have florid tuft necrosis while at the other some are partially or wholly sclerotic. The renal changes in Case 2 are very similar. Nearly all glomeruli are abnormal, necrosis being present in some while most show cellular proliferation in both the tuft and in cells lining Bowman's capsule, with the production of epithelial crescents. Some partially sclerosed glomeruli are seen, but this change is not so pronounced as in Case 1. The main point of difference from the latter case is the absence of acute necrotizing arteritis. The picture is one acceptable as the extracapillary type of subacute glomerulonephritis (Volhard and Fahr, 1914) or rapidly progressive Type 2 nephritis (Ellis, 1942), but more necroses are present in the tufts than are usually seen in this type of glomerulonephritis. There seems little doubt that there is a considerable overlap between this variety of glomerulonephritis and the kidney of polyarteritis nodosa with glomerular lesions, crescent formation and proliferative changes being present in both although more pronounced in the former while tuft necroses are more prominent in the latter. Case 3 showed a renal lesion of chronic glomerulonephritis with in addition evidence of more recent proliferative activity. Evidence of healed necrotizing arteritis was sought, but no convincing changes were found either in the kidney or elsewhere.

With regard to the lung changes, Cases 2 and 3 show the greatest similarities both in the way in which haemoptysis had been present for several weeks before death and also in the pathological picture. Both showed widespread fresh intra-alveolar haemorrhage and older changes in the form of iron-containing macrophages and organized fibrin. Case 1 showed a much more recent picture in that most of the haemorrhage was fresh although there was some evidence of older bleeding. In no case was there a necrotizing arteritis nor was there evidence of necrotizing alveolitis nor infiltration of the inter-alveolar septa by inflammatory cells as described in the series of Parkin *et al.* (1955). Haemorrhage into alveoli is one of the essential features of idiopathic pulmonary haemosiderosis, and because of the essential similarity between the lung changes in this condition and those of the cases under discussion the published cases of idiopathic haemosiderosis were reviewed to see if there was a high incidence of renal lesions.

Idiopathic pulmonary haemosiderosis was first described by Ceelen in 1931, the majority of cases occurring in childhood, although increasing numbers are now being described in adults. Halmágyi, Felkai, Sövényi, Weber, Czipott, Kovács, and Steiner (1956), Borsos-Nachtnebel (1942), Russi and Wingo (1956), and Tait and Corridan (1952) have described patients aged 40, 38, 37, and 31 years respectively, all of them being men. A further 11 recorded cases affecting people over the age of 19 years have been found. The lungs are heavily impregnated with haemosiderin contained in macrophages in the alveoli, in the septa in relation to fragmented elastica, and in the walls of blood vessels. Both the cause and the source of the haemorrhage remain obscure, the necrotizing arteritis described by Anspach (1939) being exceptional. It seems more likely that the bleeding is from the alveolar capillaries or from bronchopulmonary anastomoses as proposed by Lendrum, Scott, and Park (1950) in mitral stenosis. The aetiology has aroused much speculation, but at the present time no theory advanced has much scientific evidence to substantiate it.

Out of a total of 69 recorded cases of idiopathic pulmonary haemosiderosis, five cases have been found in which a renal lesion was present. Glanzmann and Walthard (1941) described a girl of 13 years with a characteristic history but who in addition had albumin, red blood cells, and casts in urine. At necropsy a renal picture was found which they regarded as focal embolic nephritis, glomeruli showing partial loop necrosis and increased endothelial cells. They attributed this to an attack of scarlatina at 6 years of age. Scheidegger and Dreyfus (1945) described a girl aged 1 year in whom renal changes in life were manifested by a mild proteinuria and red blood cells and casts in the urine. The kidneys showed what the authors described as focal nephritis. Propst (1955) described a focal acute to subacute glomerulonephritis in an 11-year-old boy with the usual lung picture. The description of the renal

lesions was scanty and little attention was paid to it. Bruwer, Kennedy, and Edwards (1956) described a 12-year-old boy with lung changes of idiopathic haemosiderosis and renal lesions of polyarteritis nodosa with glomerular changes. Necrotizing arteritis was found in small lung arteries and in arterioles in the sciatic nerve and testicle. The fifth case was described by Soergel (1957), the patient being a girl of 13 years whose pulmonary symptoms dated from the age of 6 years. No urinary abnormalities were described during life, but at necropsy it is stated that a focal acute glomerulitis was present but no details are given. In addition three other cases have been found in which urinary abnormalities were found during life but the state of the kidneys at necropsy was not described. Waldenström's (1944) second case, a boy of 15 years, showed proteinuria, but in the necropsy report published in a separate paper by Gellerstedt (1939) there is no mention of the kidneys. The second of three cases described by Browning and Houghton (1956) showed a pronounced albuminuria, but again there is no description of the kidneys at necropsy. In the case of a 7½-year-old boy Schuler and Flesch (1955) found red and white blood cells together with casts in the urine. At necropsy the only kidney abnormality recorded was a collection of lymphocytes in the cortex.

We have recently seen a renal and a lung biopsy from a patient with repeated haemoptysis in whom a renal lesion was present. The patient, to be described in detail by Walker and Joekes, was a young man aged 20 years who, during routine examination for the haemoptysis, was found to have proteinuria with red blood cells, white blood cells, and casts in the sediment. Renal biopsy showed a focal glomerulonephritis in which some glomeruli were normal, some showed cellular proliferation, and others were either partially or completely hyalinized. Lung biopsy showed haemosiderin-containing macrophages in alveoli.

Because of the polyarteritis nodosa in Case 1 and in the case of Bruwer *et al.* quoted above, the incidence of pulmonary haemorrhage was sought in cases of polyarteritis nodosa with glomerular lesions. In particular it was felt that cases of this disease with involvement of pulmonary arteries would be the most fruitful group to study. Rose and Spencer (1957) in a description of 31 patients with lung involvement made no mention of extensive haemorrhages such as are seen in the cases under discussion. Godman and Churg (1954), in a paper describing the necropsy findings in seven cases of Wegener's granulomatosis (1939), a condition in which pulmonary arteritis and necrotizing glomerulitis are frequently found together, did not describe extensive pulmonary haemorrhage. Such haemorrhage of the lung is, however, mentioned in one of eight cases of periarteritis nodosa with lung involvement described by Sweeney and Baggenstoss (1949), but as the paper was concerned with lung changes only it is not possible to assess the condition of the kidneys. While it seems exceptional to find extensive lung haemorrhage in polyarteritis nodosa, certain solitary case reports of this association have been found. MacMahon, Derow, and Patterson (1953) described a man of 55 years who first complained of haemoptysis and then died in renal failure. The lungs showed a fulminating panarteritis of a few medium-sized vessels with capillary congestion, haemosiderosis, and induration of the lung. The kidneys showed a focal glomerulonephritis of the type seen in polyarteritis nodosa. In a clinico-pathological conference, Mendeloff and Smith (1955) described a diffuse haemosiderosis of the lungs with no vascular lesions in a 16-year-old girl, this being associated with a renal picture resembling healed focal embolic nephritis. It was suggested that the latter picture might be considered a healed form of the microscopical type of polyarteritis of Davson *et al.* Arteritic changes in the pancreas and adrenal supported this. A recent case of Wegener's syndrome described by Thomas (1958) concerned a 40-year-old man who had scattered haemorrhages in the lung, areas of lung containing iron-filled macrophages, and acute arteritis in small lung blood vessels. The kidneys showed a necrotizing focal glomerulonephritis. In four of the seven cases of haemorrhagic and interstitial pneumonitis with nephritis described by Parkin *et al.* (1955), evidence of polyarteritis nodosa was found, but arteritic changes in the lung were found in only one case, and in that case only a solitary artery was involved. Thus it would appear that even when there is evidence of polyarteritis nodosa in a case with extensive lung haemorrhage, the latter is in all probability not caused by acute arteritis and the source of bleeding is more likely to be the alveolar capillaries or broncho-pulmonary anastomoses.

The possible relationship of the lungs under discussion to the uraemic lung described by Doniach (1947) must be considered. In patients with uraemia and left ventricular failure he described lungs showing a widespread "solid oedema" due to a fibrinous or albuminous intra-alveolar exudate. This was associated with a mononuclear cell reaction, organization of

exudate, and hyaline membranes lining alveolar ducts. The exudate was considered to result from a combination of a rise in pulmonary capillary pressure due to left ventricular failure and an alteration in capillary permeability resulting from uraemia. In some of the cases red blood cells and haemosiderin-containing macrophages were found in alveoli. In the cases of the present series all had a terminal uraemia, but in only one case (No. 3) was there definite evidence of left ventricular failure. In this case there was considerably more haemorrhage and less in the nature of albuminous exudate than the usual picture described by Doniach.

Parkin *et al.* (1955) considered the association of pulmonary haemorrhage and an acute renal lesion explicable on a basis of hypersensitivity. They drew attention to the experiments of Cannon, Walsh, and Marshall (1941) and Gregory and Rich (1946) on anaphylactic pulmonary lesions in rabbits. These experimental lung lesions, in neither of which, it should be noted, was there much haemorrhage, were compared with the type of lung found in the human in acute rheumatism as described by Gouley and Eiman (1932) and Epstein and Greenspan (1941). The so-called rheumatic lung, it might be observed, differs in no way from the lungs described by Doniach and cannot be considered specific for a hypersensitivity state. This concept of Parkin *et al.* (1955) that both lung and renal lesion can be explained on a hypersensitivity basis is attractive but on the available evidence purely speculative. The renal lesion in the three cases under discussion is of essentially the same type as that described by Parkin *et al.* and of a type usually associated with hypersensitivity, although there is nothing in the case histories indicative of any sensitizing episode. The lung lesion differs from that in the series of Parkin *et al.* in that no alveolitis is present and the slight alveolar septal changes are probably a result rather than a cause of the haemorrhage. The aetiology of the lung haemorrhage must remain obscure just as in idiopathic pulmonary haemosiderosis, a condition which resembles the lungs of Cases 2 and 3 to the point of identity.

It would appear, however, from the numerous cases quoted in the discussion that there is more than a chance association of lung haemorrhage with glomerular disease, the exact relationship not being obvious.

Summary

Three patients with severe pulmonary haemorrhage dying from renal failure are described. In all there was an extensive glomerular lesion, acute in two and chronic, with evidence of recent activity, in the third. In two of the cases there was a history of repeated haemoptyses whilst in the other overt haemoptysis occurred only during the last few days of life. Clinical evidence of cardiac failure was present in only one case. The pulmonary lesions resembled closely those of idiopathic pulmonary haemosiderosis and the incidence of renal involvement in that condition is described. There is little in the present series to suggest with certainty the nature of the association between the pulmonary and the renal lesion.

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A RENAL LESION IN ASSOCIATION WITH INFLUENZA

BY

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Between the beginning of October, 1957, and the end of January, 1958, seven cases of influenza came to necropsy at the Royal Free Hospital.

All these patients had influenzal tracheitis, bronchitis, and bronchopneumonia, and six of them died from this cause. Influenza virus A (Asian strain) was isolated from the lungs of all six. In two cases the virus was the only pathogen, but in the other four there was also a secondary bacterial invader; three patients' lungs yielded *Staph. pyogenes* and the other *Klebsiella pneumoniae*. From the lungs of the seventh patient no virus was grown, but this young woman died during a relapse of the influenza. She had suppurative pericarditis and suppurative bronchopneumonia with lung abscess in addition to the influenzal lesions of the respiratory tract; from all the suppurative lesions *Strep. pyogenes* was isolated.

The clinical features, urinary findings, treatment, and post-mortem appearances are summarized in the accompanying table. It is noteworthy that the longest interval between the onset of influenza and death was 11 days, and, in view of the renal lesions to be described, it is of interest that in each case there was a terminal phase, lasting one to three days, characterized by severe toxaemia and peripheral circulatory failure.

Morbid Anatomy and Histology

The kidneys from all cases showed varying degrees of cortical pallor. In three cases the renal papillae attracted attention at necropsy because they were conspicuously swollen and were bright red; in the other four no gross pyramidal changes were observed. Similar microscopic changes were, however, found in the kidneys of all seven patients, though they varied in degree and extent. The pelves and ureters appeared normal. Histologically varying degrees of interstitial oedema and congestion were always present in the medullae and were most marked in the papillae. As would be expected, both these changes were most noticeable microscopically in the cases in which macroscopic alteration in the papillae was visible. The vascular congestion was often patchy in distribution; where it was most intense the appearances suggested that actual stasis of the blood in the capillaries had occurred during life. In one case (Case 2) there was a very striking accumulation of primitive leucocytes in vessels throughout the medulla, and especially in the papillae; this feature was detectable in the other cases but in them was slight only.

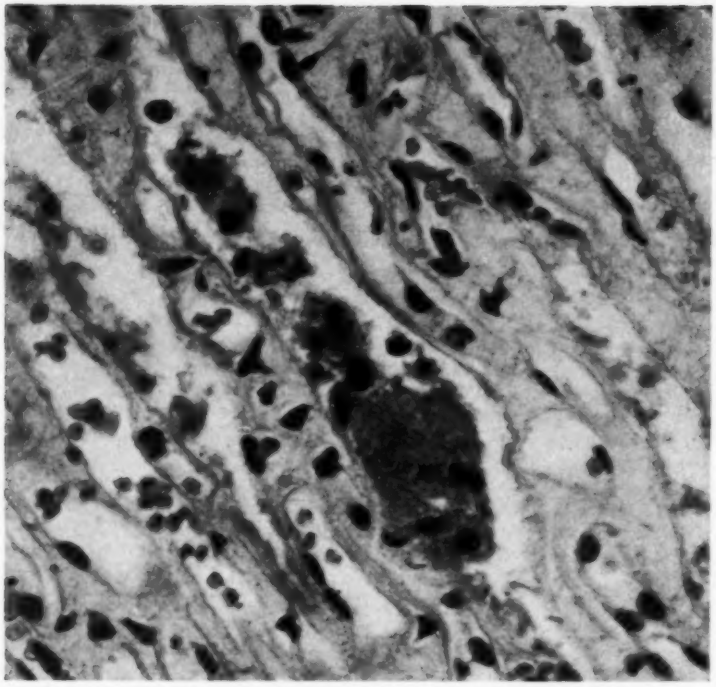
The papillae were denuded of their covering transitional epithelium; this desquamation could often be seen to cease abruptly at the bases of the papillae.

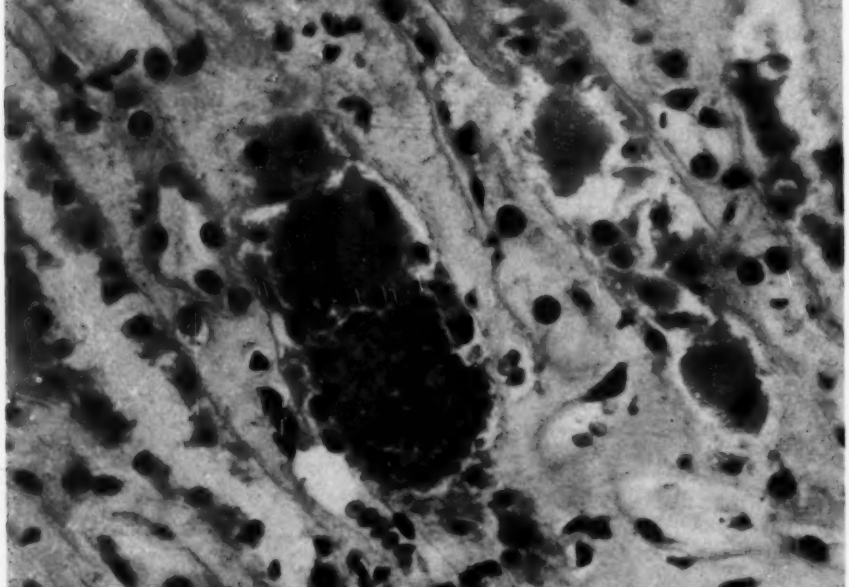
Tubular lesions were found in the medullae; they principally involved the collecting tubules, but also, to a less severe degree, the loops of Henle. Many collecting tubules had shed their lining epithelium completely, the basement membranes being left bare. It was, of course, difficult to exclude post-mortem autolysis as the cause of the tubular desquamation and it doubtless was the cause of some of it. However, close inspection of the tubules where there were some remains of epithelium still adherent or partly adherent to the basement membrane revealed changes in these cells which suggested they had undergone ante-mortem degeneration and necrosis. Many of the nuclei were pyknotic and the cytoplasm often showed a pale purple granularity which was succeeded by cytoplasmic disintegration. Epithelial cells of this kind could sometimes be seen falling away from the basement membranes and adding themselves to casts composed of similar granular material lying in the tubular lumina (Figs. 1 and 2). In other areas, tubules with intact epithelial linings contained similar casts. Several tubules which showed the epithelial changes just described also showed swelling of the basement membranes. Tubular ruptures and tubulo-venous anastomoses could not be demonstrated with certainty, but red cells could often be seen in the lumina of tubules which showed the necrotic lesions considered to be ante mortem. In places small groups of collecting

TABLE

CLINICAL AND POST-MORTEM SUMMARIES OF SEVEN CASES OF INFLUENZA

Case No.	Sex	Age	Duration of Influenza	Clinical Summary	Treatment	Post-mortem Findings	
						Morbid Anatomy	Bacteriology and Virology of Respiratory System
1	F	24	4 days	Mild case of influenza at onset. Two days before death high fever, severe cough and retrosternal pain suddenly developed, with signs of bronchopneumonia and peripheral circulatory collapse. Marked leucopenia. No response to treatment Urine: no information	Penicillin Sulphatriad Chloromycetin Erythromycin Noradrenaline Hydrocortisone Oxygen	Necropsy 17 hr. after death. Typical influenzal tracheitis, bronchitis, and bilateral bronchopneumonia Kidneys: cortices pale and slightly swollen. Medullae congested	1. Influenza virus A (Asian strain) 2. *Staph. pyogenes*
2	F	23	8 ,,	Sixteen weeks pregnant. Mild attack of influenza at onset. Two days before death considered to be convalescent. Then suddenly developed cough, high fever, and pain in back and across front of chest. Cardiac impulse not palpable; heart sounds soft and distant. Signs of bilateral bronchopneumonia. No response to treatment Urine: trace of albumin; occasional white and red blood cells; a few granular casts. No organisms Culture: sterile	Chloromycetin Hydrocortisone Gamma globulin Noradrenaline Fresh blood transfusion Oxygen	Necropsy 18 hr. after death. Trachea and bronchi denuded of epithelium and bright red. Lungs showed haemorrhagic influenzal pneumonia; small acute abscess in right upper lobe Heart: acute suppurative pericarditis; myocardium clay-coloured, small abscesses in wall of left ventricle Kidneys: renal papillae bright red and very swollen. Cortices pale and slightly swollen	1. Influenza virus not isolated 2. *Strep. pyogenes* (*Strep. pyogenes* also isolated from pericardial exudate)
3	F	19	8 ,,	Mild attack of influenza at onset. Considered to be recovering 3 days before death; thereafter she rapidly developed high fever and signs of encephalitis and bilateral bronchopneumonia. Lapsed into coma. No response to treatment Urine: no abnormal findings	Chloromycetin Cortisone Anti-staphylococcal serum Prednisone Oxygen	Necropsy 18 hr. after death. Typical influenzal tracheitis, bronchitis, and bilateral bronchopneumonia Kidneys: papillae deep red and very swollen. Cortices pale and slightly swollen	1. Influenza virus A (Asian strain) 2. No bacterial pathogens isolated
4	M	66	4 ,,	Fairly mild attack at onset, with general malaise, headache, vomiting, and slight diarrhoea. 2 days before death developed severe cough, diarrhoea, bilateral bronchopneumonia and extreme collapse No response to treatment Urine: none obtained	Penicillin Oxygen	Necropsy 56 hr. after death. Mild, pre-existing chronic bronchitis and emphysema. Severe acute purulent tracheitis and bronchitis. Haemorrhagic influenzal bronchopneumonia with suppuration in the lower lobes Kidneys: Capsules stripped with slight difficulty. Cortices pale and swollen	1. Influenza virus A (Asian strain) 2. *Klebsiella pneumoniae*
5	M	21	2 ,,	Mild onset of influenza with cold and sore throat. Severe coughing and vomiting that night. Found collapsed next day and removed to hospital. Signs of bronchopneumonia and peripheral circulatory collapse. No urine obtained. No response to treatment	Penicillin Coramine Oxygen	Necropsy 8 hr. after death. Typical influenzal tracheitis, bronchitis, and bilateral bronchopneumonia Kidneys: Medullae congested, increased pallor of cortices	1. Influenza virus A (Asian strain) 2. *Staph. pyogenes*
6	M	69	3 ,,	Mild onset of influenza; at work for first two days of illness. Then severe diarrhoea developed, followed by high fever, bronchopneumonia, and peripheral circulatory failure No response to treatment. No urine obtained for examination	Oxygen (died before any other treatment could be given)	Necropsy 19 hr. after death. Typical influenzal tracheitis, bronchitis, and bilateral bronchopneumonia Kidneys: Renal papillae deep red and very swollen. Cortices rather pale	1. Influenza virus A (Asian strain) 2. *Staph. pyogenes*
7	M	60	11 ,,	History of chronic bronchitis, with recent attack of pneumonia. Influenza mild until 24 hr. before death, when high fever, signs of bronchopneumonia, and peripheral circulatory failure developed. No response to treatment. Urine normal at onset of influenza. Not examined later	Penicillin Achromycin Aminophylline A.C.T.H. Oxygen	Necropsy 36 hr. after death. Chronic bronchitis and severe hypertrophic pulmonary emphysema. Typical influenzal tracheitis, bronchitis, and bronchopneumonia Kidneys: Uniformly congested	1. Influenza virus A (Asian strain) 2. No bacterial pathogens isolated





FIGS. 1 and 2.—Collecting tubules in renal medullae showing progressive cell necrosis and disintegration of purplish, granular cytoplasm of the epithelium with formation of casts of similar material within the tubular lumina. Haematoxylin and eosin, ×540.

tubules attracted attention under the low power of the microscope because they were filled with loosely-arranged masses of pleomorphic epithelial cells. High-power examination showed some of the cells to have pyknotic nuclei and granular, disintegrating cytoplasm; others were angular in shape, had large hyperchromatic nuclei, and appeared to be regenerating. Mitotic figures in the regenerating cells were very scanty, but a fair number of binucleate cells were seen.

Tubules showing the appearances just described were numerous in only three cases, though lesser degrees of similar changes could be seen in the collecting tubules in all cases. At this point it may be well to remark that many tubules were present in all the kidneys in this series in which separation of the epithelium into the lumina was considered to be the result of fixation and post-mortem artefacts only; these included all tubules in which the lining epithelium had separated *en masse*, forming coherent epithelial rings inside tubules, and all tubules in which small rows of desquamated cells adhered together in groups of two, three, four, or more cells. Tubules affected in this way by autolysis differed greatly in appearance from those described above, in which the lumina contained a mixture of degenerating and regenerating epithelial cells.

Milder tubular lesions were observed in the loops of Henle in all cases; frank necrosis was absent, but in places the lining cells had swollen and also showed increased cytoplasmic eosinophilia. The loops of Henle contained many hyaline eosinophil casts.

In four of the seven cases there were varying amounts of calcium deposited in the renal tubules (Cases 3, 5, 6, and 7). Two of these patients were elderly, but the other two were young individuals. Calcium deposition was confined to degenerate epithelium in the medullae in three instances, but in the fourth patient, a man aged 60 years, there was some calcification of the epithelium of the cortical tubules. Post-mortem autolysis was advanced in the renal cortices in this case and appeared to be uniform throughout the cortical tubules which showed no evidence of focal or diffuse necrosis.

No ante-mortem lesions more severe than cloudy swelling were detected in the cortical tubules of any patient in the series. The capsular spaces of the Malpighian corpuscles were somewhat dilated and contained small amounts of protein. The glomeruli were essentially normal in all cases, except for the presence of a few sclerosed glomeruli in very occasional pyelonephritic scars in the cortices of the older individuals. Apart from this, none of the kidneys in the series showed any evidence of chronic renal disease and there were no significant hypertensive changes.

Discussion

Renal lesions in association with influenza have occasioned little detailed comment in the medical literature. Winternitz, Wason, and McNamara (1920) in their classic work on the pathology of this infection stated that cloudy swelling of the parenchymatous organs was marked and in many cases was associated with cellular necrosis in the liver and kidneys; the necrosis was usually focal. Mitotic figures in the cells of the convoluted tubules, possibly indicating previous damage, were occasionally seen. Acute nephritis, previously reported, was not seen in this large series of cases.

Opie (1928), in a review of the pathology of influenza, stated that parenchymatous degeneration of kidney tubules was often conspicuous, but details of the site of the tubular lesions were not recorded. Earlier, Symonds (1918) reported a series of 22 cases of influenza, 11 of them fatal, in young soldiers; well-marked signs of renal disease complicated the respiratory infection. Urine examination revealed abundant albuminuria and numerous casts, but gross haematuria was not a feature and small numbers of red cells were found in only three of the cases. At necropsy none of the kidneys showed any changes except some increased adherence of the capsules; histological examination revealed congestion and varying degrees of tubular degeneration, but autolysis was too advanced to permit any detailed studies of the tubular lesions. In our series of seven cases the urinary findings are poorly documented and it has not been possible to correlate them with the renal pathology. Several of the patients, however, were admitted *in extremis* and produced little or no urine; those in hospital passed suddenly into a short terminal phase and again no urine was obtained.

Lucke, Wight, and Kime (1919) reviewed the pathology and bacteriology of 126 fatal cases of influenza and discussed the renal findings in considerable detail. Ninety per cent. of the kidneys examined were somewhat enlarged, the cut surfaces were moist and bloody, the cortices were usually swollen. "In every instance parenchymatous changes, usually of mild degree, but often quite severe were observed." The histological findings included moderate oedema of the interstitial tissue, swelling of the capsular epithelium, and deposition

of granular eosinophil material in capsular spaces and tubular lumina. The tubular epithelium was swollen, ragged in outline and often vacuolated; nuclear changes ranged from poor staining to complete disappearance. These authors remarked that tubular damage was seldom uniformly distributed, groups of necrotic tubules being seen in close proximity to normal ones. They found that "in every case the convoluted tubules were more affected than the straight tubules," and here their observations differ from the findings in the present series of cases. Nuzum, Pilot, Stangl, and Bonar (1918), Blanton and Irons (1918), Symmers (1918), Dever, Boles, and Case (1919), Plenge (1952), and Fischer (1957), though dealing in much less detail with renal lesions in influenza than did Lucke *et al.* (1919), agreed with the latter authors as to their nature and distribution.

In publications stemming from the recent influenzal pandemic little has been written about renal lesions. A combined study group (*Brit. med. J.*, 1958), reviewing some aspects of the recent epidemic of influenza in Dundee, record pathological observations on 24 necropsies and state that "a feature of nine cases was extreme pallor of the kidneys." In three of these cases there was microscopical evidence of bilateral necrosis of proximal tubules, but in the remaining six cases only cloudy swelling and post-mortem autolysis was found. Neilson (1958), reporting three sudden deaths from fulminating influenza, found intense congestion of the kidneys. Roberts (1957) records that "no significant change was found in any of the viscera apart from the respiratory system" in nine fatal cases of influenza in previously healthy individuals. Giles and Shuttleworth (1957), reviewing the post-mortem findings in 46 influenzal deaths, found long-standing, chronic renal disease in four patients. One young girl was admitted to hospital with influenza and pre-eclamptic toxaemia of pregnancy; she developed pneumonia and died in the first stage of labour two days later, and the kidneys showed oedema and relative avascularity of the glomeruli. Darke, Watkins, and Whitehead (1957) in their fatal case of influenzal pneumonia found only toxic changes in the liver in addition to the respiratory lesions. Herrmann, Ogura, Johnson, Toll, and White (1958), in a detailed review of 23 cases of sudden death reported to the Denver coroner in October, 1957, and diagnosed at necropsy as being caused by influenza, mention briefly that they consistently observed "mild degenerative changes in such organs as the heart and kidneys."

The particular interest of our material is that the renal lesions were largely concentrated in the medullae. Some of the histological changes observed were undoubtedly autolytic, and others, such as minor degrees of tubular calcification, almost certainly antedated the final illness, but it is difficult to account for all the changes observed on these grounds. Our cases all showed uniform cloudy swelling without necrosis in the epithelium of Bowman's capsule and the proximal tubules; this is in accordance with the experience of previous authors. It seems probable that attention was then drawn to the focal lesions in the lower tubules by the striking changes in the papillae which could be seen on macroscopic examinations of the kidneys from some of the cases.

In the light of the evidence at present available to us, we do not consider it justifiable firmly to ascribe the distal tubular damage to influenza in itself. Unfortunately, renal tissue for virological studies was not taken at necropsy, and viral inclusions are not demonstrable in tissues infected by influenza. The use of embryonic human and adult monkey kidney tissue viruses (Mogabgab, Green, and Dierkhising, 1954; Mogabgab, Green, Dierkhising, and Phillips, 1955) does, however, suggest that these organisms may have some affinity for renal epithelium.

It is improbable that the renal lesions were due to direct bacterial infections, because no organisms were identified in histological preparations and the morphological changes were not those of acute bacterial inflammation. Moreover, two of the cases had no secondary bacterial invasion of the respiratory tract.

The very diversity of medical treatment makes it unlikely that the kidney lesion is attributable to drug therapy. Another possibility is that the changes in the renal medullae might be the result of toxaemia or peripheral circulatory failure, which all our cases suffered during life, or to a combination of these two factors. Toxic changes, however, usually affect the proximal renal tubules rather than the distal ones and the focal ischaemic necroses of "shock" have been shown by the microdissection studies of Oliver, MacDowell, and Tracy (1951) and others to be distributed to all parts of the nephrons. Moreover, we have carried out a review of the routine histological sections of the kidneys from 75 recent necropsies performed in our hospital, and it is noteworthy that, although these kidneys included many from patients who had suffered severe degrees of toxaemia or shock or both, only one case was found in which the

medullary lesions were identical with those seen in the influenzal material. The certified cause of death in this case was lobar pneumonia and hepatic cirrhosis, but reassessment of the clinical, bacteriological, and post-mortem findings and of the available histological material strongly suggested that the patient had, in fact, died of fulminating influenzal pneumonia.

Thus the pathogenesis of the renal lesion remains obscure and its specificity and significance are largely undetermined. Before speculating further it is important to know the findings of other observers who have carried out necropsies on cases of influenza from the recent pandemic.

Summary

Renal changes in seven fatal cases of influenzal pneumonia, due to virus A (Asian strain), are reported. The morbid anatomy and histology of the renal lesions, which were principally localized to the medullae of the organs, are described and illustrated.

The literature on renal pathology in influenza is briefly reviewed; in several previous reports only cortical changes are described. It has not been possible to form a decided opinion as to the aetiology of the medullary lesions.

We thank the several clinicians at the Royal Free Hospital for allowing us to use their case notes, colleagues in the Department of Pathology for technical assistance, Mr. R. R. Phillips for the photomicrography, and the Routine Diagnostic Laboratory, Colindale, for virological studies.

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J. clin. Path. (1959), 12, 286.

STAPHYLOCOCCAL CYSTITIS IN A GYNAECOLOGICAL WARD

BY

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(RECEIVED FOR PUBLICATION JULY 5, 1958)

Cross-infection of wounds in hospital is well recognized. Similar infection of the urinary tract received less attention until Ørskov (1952, 1954) showed that many urinary infections in certain Copenhagen hospitals were due to the same organism. She was able to identify the *Klebsiella* strains by their biochemical and antigenic properties. Kirby, Corpron, and Tanner (1956) mention instances in which cross-infection by antibiotic-resistant staphylococci outside the urinary tract was proved by phage-typing, but point out that there is no simple way of labelling coliform organisms so as to identify cross-infecting strains of the urinary tract. They described infections of the urinary tract with coliform organisms which were resistant to antibiotics. When such organisms were encountered in patients without previous antibiotic therapy, the cases were regarded as being due to cross-infection. The methods of sterilization of the catheters and irrigation solutions were satisfactory, and they suggested, therefore, that the organisms originated in the naso-pharynx of the hospital staff or from heavily contaminated blankets.

Dutton and Ralston (1957), dealing with urinary tract infection in a male urological ward, found evidence of cross-infection. Winchester bottles for collecting urine from patients with indwelling catheters, urinals, nurses' hand towels, nurses' hands, ward air, and dust were found to carry organisms similar to those infecting the urinary tracts of the patients.

This report deals with an outbreak of *Staphylococcus aureus* cystitis in a gynaecological ward. Phage-typing showed that the organisms were probably all derived from a common source.

Cases of Cystitis

In August, 1955, it was noticed by Dr. P. N. Coleman that the frequency with which he isolated *Staphylococcus aureus* from the urine of patients in the ward was increasing.

The histories of all patients admitted to the ward from the middle of May to the end of October were therefore examined. Thirty-seven of 138 had cystitis. In each case the disease was characterized by frequent dysuria and pyrexia. The bacteriological results are given in Table I, from which

TABLE I
INCIDENCE AND TYPE OF CYSTITIS

Total No. of Patients	*Staph. aureus* Phage Type 7	*Staph. aureus* Not Typed	*Bact. coli*	Sterile
138	12	8	14	3

it will be seen that 20 of the infections were due to *Staphylococcus aureus*. All 20 strains were resistant to penicillin and streptomycin, very sensitive to chlortetracycline, and slightly sensitive to chloramphenicol. Twelve of the 20 strains were phage-typed by Dr. E. H. Gillespie of the Public Health Laboratory, Sheffield, and all 12 were phage-type 7.

Eighteen of the 20 patients infected with *Staphylococcus aureus* had previously passed sterile urine. Of the two patients who had not, one had previously yielded *Staphylococcus aureus* (not phage-typed) and is not included as a case of cross-infection. From the other patient, *Ps. pyocyanea* was isolated from an earlier urine.

Search for Source of Infection

Surgeons.—As all the infections occurred after operations, the question arose whether they were associated with one particular surgeon. The fact that surgeon A had nine cases and surgeon B had 10 proved that this was not so. Neither surgeon carried the epidemic strain in his nose. The relation was then considered of the incidence of cystitis to the type of surgical operation used (Tables II and III). It was evident that the incidence of cystitis was greater after vaginal than after abdominal operations.

TABLE II

INCIDENCE OF *STAPHYLOCOCCUS AUREUS* CYSTITIS RELATED TO NATURE OF OPERATION

No. of Cases	Vaginal Repairs	Vaginal Hysterectomies	Examination under Anaesthesia and Dilatation and Currettage	Abdominal Operations
19	9	4	1	5

TABLE III

TYPE OF OPERATION AND INCIDENCE OF INFECTION

	Staphylococcus aureus	Coliform Organisms	No Infection
Vaginal operations ..	14	11	7
Abdominal ,, ..	5	1	27

It therefore appeared that infection was introduced either in the theatre or in the ward during the post-operative routine. The surgeons themselves carried out catheterization in the theatre; as the surgeons were shown to be free from *Staphylococcus aureus* type 7, it was concluded that infection was introduced in the ward. The finding of the epidemic strain in two specimens of urine without any pus suggested that the organism was introduced during catheterization. The pre-operative and post-operative ward routines were next investigated.

Pre-operation Routine.—In May, the month before the increase of cases of cystitis, all patients of surgeon B undergoing vaginal operations had a penicillin-streptomycin pessary inserted in the vagina for three nights before operation. This became routine for both surgeons in mid-September. The pessaries were inserted by a nurse whose hand was protected with a boiled, dried, and powdered but non-sterile glove. The gloves were hung to dry on a line in the sterilizing room adjacent to the ward, from the dust of which the epidemic strain was isolated. Six special blankets for use on the theatre trolley were kept for these patients; they were removed in the anaesthetic room and never taken into the theatre.

Catheterization Routine.—Catheters were passed by the staff nurse or a senior nurse who had been adequately instructed. Technique was conventional except that gloves were not worn. The catheter lubricant consisted of tragacanth jelly with phenyl mercuric nitrate. In May, 1955, a change was made; this mixture was steamed for half an hour, then 150 mg. of dihydrostreptomycin was added to 120 g. of the original mixture.

After operation, patients either had an indwelling catheter for 48 hours and/or were catheterized eight-hourly until the residual urine was less than 2 oz.

Nasal Carriers.—Nasal swabs were taken from the doctors, nurses, and ward cleaners. Three nurses harboured the epidemic strain, staff nurse M who performed most of the catheterizations and two nurses who worked for a period of only one week in the infected ward. Five infections occurred during the absence of nurse M. It was therefore assumed that she was a recipient and not the reservoir of the epidemic strain.

Blankets and dust were sampled and *Staphylococcus aureus* of the epidemic type was isolated from two out of four blankets, four out of five samples of ward dust, and from dust in the sterilizing room.

Preventive Measures

The ward and side rooms were washed with soap and water from floor to ceiling and then re-decorated. Blankets were treated with "lissapol" and "cirrasol OD" (Blowers and Wallace, 1955).

The catheterization routine was modified so that masks and gowns were worn, and hands were scrubbed for at least three minutes and dried on a sterile towel before sterile gloves were put on. Great care was taken with the preparation of the patient; "dettol" was being used to swab the vulva. No catheter lubricant was used. The name of the nurse performing the catheterization was recorded each time. Penicillin-streptomycin pessaries ceased to be used.

Results

During the next 17 months only one case of staphylococcal cystitis occurred after 384 similar operations.

Discussion

The pattern of the sensitivity of the organism to antibiotics was the same for all the organisms that were typed, namely, resistant to penicillin and streptomycin, very sensitive to chlortetracycline, and slightly sensitive to chloramphenicol. This supports the belief that we were dealing with a single strain of *Staphylococcus aureus*. How it was introduced it is impossible to say, as investigations were not begun until four months after the first diagnosis of staphylococcal cystitis. By this time the organism was generally distributed in the dust and blankets, although it was isolated from only a few members of the nursing staff.

The possibility that the staphylococcus was in the catheter lubricant must be considered. Although no tests were made, the method of preparation and sterilization makes this most unlikely. It may have come from the hands of the nurses catheterizing the patients, but there was no obvious relation between the nurses carrying the epidemic strain and the patients infected.

The original suggestion made by Kirby *et al.* (1956) that infection in such cases is due to contamination of sterile catheters by organisms from the blankets and dust at the time of catheterization must be considered the most likely

explanation. It is hardly surprising that the trouble arose after the introduction of penicillin-streptomycin pessaries, because any substance that removes the normal bacterial flora leaves the treated area ripe for colonization by antibiotic-resistant organisms, including staphylococci.

Summary

An account is given of staphylococcal cystitis due to cross-infection. It is suggested that the infection was due to catheterization and was favoured by the use of antibiotic pessaries. The organisms probably came from the blankets and dust. Measures taken to reduce this source of infection were successful in controlling the outbreak.

I wish to thank Mr. J. S. Hovell and Mr. K. Girgis for their help and co-operation which made this investigation possible, and Miss Yarker, the ward sister, for her valuable assistance.

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Correction

In the paper "Complexiметric Determination of Calcium in Pathological and Physiological Specimens," by D. N. Baron and Joyce L. Bell (*J. clin. Path.*, 1959, **12**, 143), there is an error in the last paragraph of column 2 on page 143. The second sentence should read:

"Then 1 ml. urine is added to the first tube and 0.2 ml. to the second tube."

J. clin. Path. (1959), **12**, 289.

A CRITICAL EVALUATION OF RED CELL AND PLASMA VOLUME TECHNIQUES IN PATIENTS WITH CIVILIAN INJURIES

BY

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There is now much evidence to suggest that the major cause of red cell disappearance following trauma is haemorrhage. After severe skeletal injuries, an abating but often clinically important bleed continues for one to four days (Clarke, Davies, Fisher, and Topley, 1959). It can be partly appreciated by the nature of the injury and a judgment of the external blood loss and internal swelling, the early swelling often being entirely due to haemorrhage. The local bleeding can also be assessed indirectly by an estimation of the amount of blood lost from the general circulation. The major cause of "wound shock" is now realized frequently to be a low blood volume, itself caused by blood loss unreplaced by blood transfusion. The effect of the low blood volume can therefore be used as a crude guide to possible haemorrhage. The level of the pulse and blood pressure (Fisher, 1958) and the changing level of the external jugular vein are of limited value as a guide to total blood volume. By these multiple approaches the experienced surgeon often assesses the timing and degree of haemorrhage to within ½ to 1 litre of whole blood (10–20% of the normal blood volume of a man 170 cm. tall). But there are occasions where all these guides are too crude; a haemorrhage of a litre or more of blood could be overlooked; and the emergency management of the injury requires a greater precision in the assessment of haemorrhage. It is under these conditions that the laboratory can often assist by measuring the circulating red cell volume. This red cell volume estimate can be compared with the average normal red cell volume estimated from the height. A "red cell volume estimate of haemorrhage" (Topley, Fisher, Davies, and Clarke, 1959) can be obtained by subtracting the volume of blood found to be remaining in the patient from that assumed to be present before injury and adding the volume of blood transfused. Similarly, continuing haemorrhage can be measured by repeated estimates of the red cell volume. Grant and Reeve (1951, e.g., p. 113) emphasized the great clinical value of such estimates in the management of war injuries, especially after operation. Experience in this hospital since the war has shown that repeated red cell volume estimates have made clinical sense when compared with the evidence obtained from the volumes of blood transfused, the evidence of local bleeding, and the later haemoglobin levels (Topley *et al.*, 1959). The evidence suggests that the "red cell volume estimates of haemorrhage" are usually measuring blood loss to the nearest ½ or 1 litre of blood. During the last few years, such red cell volume estimates in selected patients have been used as one part of the evidence on which blood transfusion policy is based. There is no doubt that ^{32}P or ^{51}Cr estimations of red cell volume are of considerable clinical assistance in assessing haemorrhage in selected injuries, especially pelvic or chest injuries, severe multiple injuries, or injuries where there is some conflicting evidence, using the clinical criteria outlined above. It is likely that surgeons will increasingly ask for such a red cell volume estimate of haemorrhage (Topley *et al.*, 1959). For this reason the present paper discusses critically the technique of the red cell volume estimate in patients during the hours and days after injury.

Methods of estimating circulating red cell and plasma volume have been reviewed in terms of their technical details, their accuracy in normal individuals, and the changes measured in disease (Reeve, 1948; von Porat, 1951; Gregersen, 1951; Mollison, 1956; Wiklander, 1956; Brown, Hopper, and Wennesland, 1957). Much less evidence is available on the inaccuracies that may be found when disease is present.

We report here techniques for estimating circulatory volume and an assessment of factors which may cause inaccuracy. The laboratory findings during the hours and days after injury have been compared with those obtained subsequently in the same patients and with those reported by others for normal individuals. In some patients either simultaneous or sequential estimations by different methods have also been compared.

Methods

Red cell or plasma volume estimations have been made on 86 patients suffering injury from road traffic, domestic, or industrial accidents. At the start of this study in 1953 investigations were carried out between one and four days after injury, when the circulation was probably approaching normal. More recently, with experience of the method, and its more certain clinical value, estimations have been made during the first few hours after injury. During the major part of this study, the first red cell or plasma volume estimation was made between one and 24 hours after injury, and repeated during the ensuing days. Where possible a follow-up estimation was made three to 12 months later. In order to minimize radiation dosage, the total number of estimations on one patient did not exceed four unless there were strong clinical indications for further estimations.

Red Cells Labelled with Radioactive Phosphorus (^{32}P).—The method was that described by Chaplin (1954) with the following minor modifications:— The heparinized blood from the patient was labelled with 1 to 5 μC of ^{32}P for adults instead of 10 to 15 μC. For children the dose was reduced according to their expected red cell volume. After incubation the labelled cells were centrifuged at 2,500 r.p.m. for three minutes instead of six to eight minutes. The labelled cells were transferred to a special long syringe* containing between 8 and 10 ml. and calibrated gravimetrically without the needle. During all manipulations of the red cells strict aseptic precautions were observed. Intravenous injection was usually directly into a vein, but sometimes, particularly during the first few hours after injury, into a blood transfusion drip tubing. If there was the slightest doubt about the efficiency of the intravenous injection, the estimation was abandoned. Samples were taken from a remote vein between nine and 120 minutes after injection. The first sample was always taken within 20 minutes. The red cell volume (R.B.C.V.) was calculated from this 9–20 minute sample using the following formula:

$$RBCV = \frac{A \times B \times C \times VH}{D \times E \times 100}$$

Where A = volume of labelled cells injected
B = dilution of standard
C = count of standard
VH = venous haematocrit corrected for trapped plasma (Chaplin and Mollison, 1952)
D = dilution of sample
E = count of sample after correction for loss of radioactivity from the circulation between injection and sampling by assuming the rate of disappearance to be 0.1% per minute

*From the Medical Supply Association.

With each red cell volume estimation, after washing the labelled cells with plasma saline, the supernatant from the final red cell suspension was removed for counting. The observed supernatant count was compared with the total amount of radioactivity injected, to check that at least 99% of the injected radioactivity was contained in the red cells. On 10 occasions red cells stored in acid-citrate-dextrose (A.C.D.) solution for transfusion purposes were labelled in the same manner.

All solutions containing ^{32}P were counted in a type M6 liquid G-M counter after lysis of the red cells with saponin. The random distribution error of counting was always less than $\pm 2\%$ (2,500 counts) and often less than $\pm 1\%$ (10,000 counts). The stock solution of ^{32}P was always resterilized within 20 minutes of opening.

Red Cells Labelled with Radioactive Chromium (^{51}Cr).—Red cells either from the patient or from a bottle of blood about to be transfused were labelled by the method of Mollison and Veall (1955) with 5 to 50 μC according to the expected red cell volume of the patient; 25 μC was commonly used in adults. Intravenous injection and sampling were exactly as for ^{32}P-labelled cells. During the preparation of the labelled cells the extracellular radioactivity was removed by three washes with plasma saline for the first 50 experiments, but only two washes were performed on the remaining 60 occasions. The corresponding supernatant radioactivity values have been determined as for ^{32}P.

All samples containing ^{51}Cr were counted as 10 ml. of solution in a scintillation counter having a $\frac{1}{2}$-in. diameter cylindrical thallium activated sodium iodide crystal (sensitivity for ^{51}Cr=9,530 counts/minute/μC).

The calculation of the red cell volume was similar to that of ^{32}P, with the exception that no correction was made for loss of radioactivity from the labelled cells between the time of injection and sampling.

Plasma Proteins Labelled with T-1824 (Evans Blue).—Following the method of Reeve (1948), 1.2 ml. of a 2% solution of T-1824 in distilled water was added to 10 ml. of sterile saline immediately before use. About 10 ml. of this solution was drawn into the syringe and the remainder used as the standard. About 20 ml. of blood was taken for a plasma blank and for preparing the standard before the dye was injected. For some years three blood samples were taken at 10-minute intervals after injection from a remote vein. More recently only a single sample has been taken. After determination of the haematocrit the plasma was separated from each of the samples. A standard was prepared containing a known volume

of the original T-1824 solution in a known quantity of the patient's undyed plasma. The optical density of the standard and the plasma was measured at wavelengths of 540, 620, and 680 mμ respectively, using a "unicam" SP600 spectrophotometer. The patient's undyed plasma was used as a blank against which the optical density of the dyed sample was compared. The relationship between the low optical density of the dye in plasma at 680 mμ to its high optical density at 620 mμ has been plotted for each batch of dye. The 680 mμ T-1824 optical density can be read off the graph on the assumption that all the 620 mμ optical density is due to T-1824. Since the extra opacity has almost the same absorption at 620 mμ and 680 mμ an appropriate correction can be calculated for the T-1824 absorption at 680 mμ. Thus:

T-1824 O.D. 620 mμ = O.D. 620 mμ − O.D. 680 mμ + T-1824 O.D. 680 mμ, where O.D. = optical density.

The 540 mμ reading was used to check that the level of haemoglobin was not sufficient to affect the 620 mμ reading. The plasma volume (PV) was calculated from the following equation.

$$PV = \frac{F \times G \times H}{J}$$

Where F = volume of T-1824 solution injected
G = dilution of standard
H = T-1824 optical density of standard
J = T-1824 optical density of sample after correction for the loss of dye from the circulation between injection and sampling by assuming the rate of disappearance to be 0.1% per minute

Human Albumin Labelled with Radioactive Iodine (^{131}I).—Human albumin was labelled with ^{131}I to a level of 1 μC per mg. of albumin and not more than one atom of iodine per molecule of albumin by the following modifications of the technique of Veall, Pearson, and Hanley (1955). The iodine was liberated from the iodide-iodate mixture with N sulphuric acid instead of N hydrochloric acid, and the yield of labelled albumin was improved by washing the resin column with 0.5 to 1.0 ml. unlabelled albumin after the radioactive material had passed down the column.

For a plasma volume estimation, 3–5 μC of labelled albumin solution was diluted with saline before injection. Blood samples were taken from a remote vein usually 15, 30, and 45 minutes after injection. The specific radioactivity of the plasma was determined either in separated plasma or in whole blood with correction for the haematocrit. The radioactive standards were prepared by diluting a sample of the injected material with distilled water containing 0.05% potassium iodide and 1% unlabelled albumin to minimize the loss of active material on glass surfaces (Reeve, 1957).

The plasma volume was calculated from the dilution of the radioactive material using a formula analogous to that used with labelled red cells. The ^{131}I was usually counted in a liquid scintillation counter, as this equipment has a greater sensitivity to the γ radiation of ^{131}I than the M6 liquid counter has for the β radiation emitted by this isotope.

Other Methods and Calculations.—The venous haematocrit was estimated by centrifuging heparinized venous blood in Wintrobe tubes for either 30 or 55 minutes at 3,000 r.p.m. in a centrifuge with head radius 15 cm. Appropriate corrections for trapped plasma were applied (Chaplin and Mollison, 1952).

The "blood volume" has been taken as the red cell volume plus the plasma volume as calculated above when both have been measured. The blood volume has also been calculated from the venous haematocrit and either the red cell or plasma volume. An adjustment has been made for the difference between the body and venous haematocrits.

$$\text{The body haematocrit} = \frac{\text{red cell volume}}{\text{red cell volume} + \text{plasma volume}}$$

An average normal ratio between the body and venous haematocrits of 0.9 has been assumed, so that:

$$\text{Blood volume} = \frac{\text{red cell volume} \times 100}{0.9 \times \text{venous haematocrit}}$$

$$\text{or } \frac{\text{plasma volume} \times 100}{100 - (0.9 \times \text{venous haematocrit})}$$

Similarly the red cell volume has been calculated indirectly from the plasma volume and the venous haematocrit.

$$\text{Red cell volume} = \left(\frac{\text{plasma volume} \times 100}{100 - (0.9 \times \text{venous haematocrit})} - \text{plasma volume}\right)$$

Counting Mixtures of Two Radioactive Isotopes.—It was possible to use ^{32}P- and ^{51}Cr-labelled red cells simultaneously due to the differing radiation characteristics of these isotopes. The pure β radiation of ^{32}P is efficiently detected by the G-M counter and not detected by the scintillation counter, whilst the γ and *x* radiation of ^{51}Cr is efficiently detected by the scintillation counter but makes only a slight contribution (less than 1%) to the counts in the G-M tube.

The use of ^{51}Cr and ^{131}I necessitated a correction factor for the γ radiation of one isotope interfering with that of the other. The usual procedure was to make the estimations sequentially, the ^{131}I albumin being injected after the 15-minute sample for the ^{51}Cr red cell volume estimation had been taken. On a few occasions the isotopes were injected simultaneously, and then the γ activity of a sample of plasma (^{131}I alone) was subtracted from the γ activity of whole blood (^{51}Cr and ^{131}I) with appropriate correction for the haematocrit.

Results

An assessment of factors which may cause inaccuracy will first be described, the observations within 48 hours of injury being compared with

those made later. In the second section will be given the results of simultaneous estimations using various pairs of methods.

Possible Causes of Inaccuracy.—Certain factors may cause inaccuracy, namely, the radioactivity level of the supernatant, and the adequacy of intravascular mixing and survival of the labelled material.

Radioactivity Level of the Supernatant.—The radioactivity in the plasma saline in which the labelled cells were suspended (" supernatant ") has been expressed as a percentage of the total quantity of radioactivity injected (Table I), and

TABLE I

RADIOACTIVITY LEVEL OF PLASMA SALINE (SUPERNATANT) IN WHICH THE LABELLED CELLS WERE SUSPENDED EXPRESSED AS PERCENTAGE OF INJECTED RADIOACTIVITY

No. of Washes	Isotope	No. of Observations	Mean Percentage in Supernatant	No. of Observations Over 1%	Observations Over 1% as % Total Observations
Patient's cells labelled during the first 48 hours					
3	^{32}P	107	0·57	14	13
3	^{51}Cr	35	0·27	1	3
2	^{51}Cr	28	0·40	2	8
Patient's cells labelled after the first 48 hours					
3	^{32}P	175	0·57	16	9
3	^{51}Cr	6	0·26	0	0
2	^{51}Cr	5	0·31	0	0
Stored cells labelled					
3	^{32}P	10	3·3	10	100
3	^{51}Cr	50	0·28	1	2
2	^{51}Cr	25	0·41	1	4

the results obtained during the first 48 hours after injury compared with those obtained later. A comparison is made between different isotopes, the source of the red cells that were labelled, and the number of washes with plasma saline. It can be seen that the labelling of red cells stored in A.C.D. solution with ^{32}P was unsatisfactory, as the radioactivity in the supernatant was always more than 1% of the total in the injected red cell suspension. When the patient's cells were labelled with either ^{32}P or ^{51}Cr there was no difference between the mean values during the first 48 hours and those made later. There were, however, significantly more results above 1% when ^{32}P was used (30/282 or 11%) than when ^{51}Cr was used (3/74 or 4%) ($\sigma=0.575$, $t=3.30$, $P=0.001$).

The rate of leakage of ^{51}Cr from labelled stored red cells has been investigated *in vitro* as shown in Table II. Standing at room temperature for up to four hours produced no excessive leakage except when the cells had been stored for more than four weeks in A.C.D. solution.

TABLE II

RATE OF LEAKAGE OF ^{51}Cr FROM LABELLED CELLS EXPRESSED AS A PERCENTAGE OF TOTAL ACTIVITY INITIALLY IN THE CELLS

Time after Labelling	Age of Stored Cells			
	0 Days	12 Days	22 Days	30 Days
0 hour	0·20	0·40	0·20	0·65
1 ,,	0·36	0·49	0·34	0·73
2 hours	0·43	0·62	0·52	1·20
3 ,,	0·69	0·98	0·53	1·20
4 ,,	0·79	0·87	0·59	1·12

Adequacy of Intravascular Mixing and Survival of Labelled Material in the Circulation.—The changing levels of radioactivity in the circulation have been plotted for each patient as the concentration of label per unit volume of red cells or plasma (log. scale) against the time after injection (linear scale). Extrapolation of the best straight line through these points to zero time gives the theoretical initial concentration (100%), and other observations are expressed as a percentage of this value. The mean of all the percentages at each time interval after injection together with the standard deviations are plotted in Figs. 1a, 1b, 1c, and 1d for each type of label and for observations made before and after 48 hours of injury. Fifty-four estimations on 51 patients have been made with ^{51}Cr, 97 estimations on 52 patients with ^{32}P, and 79 estimations on 32 patients with T-1824. The best straight line has been drawn through the mean values to indicate the average rate of disappearance. The mean rate of disappearance of labelled red cells during 10 to 60 minutes after injection was always greater during the first 48 hours after injury compared with that observed later, but the rate never exceeded 0.3% per minute.

It can be seen (Table III) that with ^{32}P, ^{51}Cr, and corrected T-1824 only one out of 189 results taken between nine and 15 minutes after injection varied from the mean value by more than ±15%. With ^{32}P-labelled patients' cells and ^{51}Cr-labelled patients' or stored cells, the number of observations outside these limits increased as the time between injection and sampling increased. More of the ^{32}P observations exceeded the ±15% limits when they were made later than 48 hours after injury compared with earlier observations. With ^{51}Cr neither the time after injury nor the source of the cells appeared to affect the scatter.

With T-1824 the improvement after correction for extra opacity using the 680 mμ reading is very marked. No observation then exceeded ±15% of the mean value, whereas without

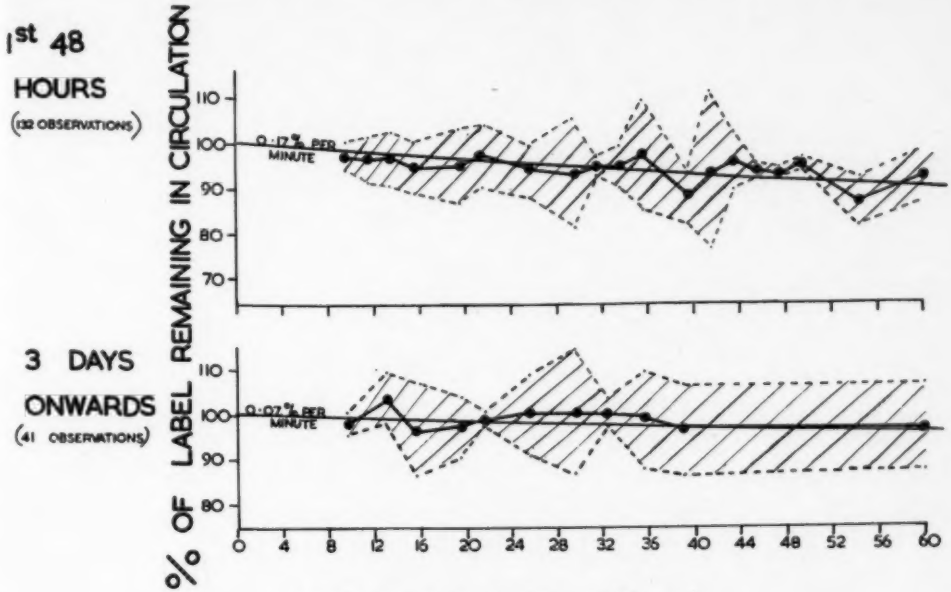

FIG. 1a.—The mean rate of disappearance of ^{51}Cr.

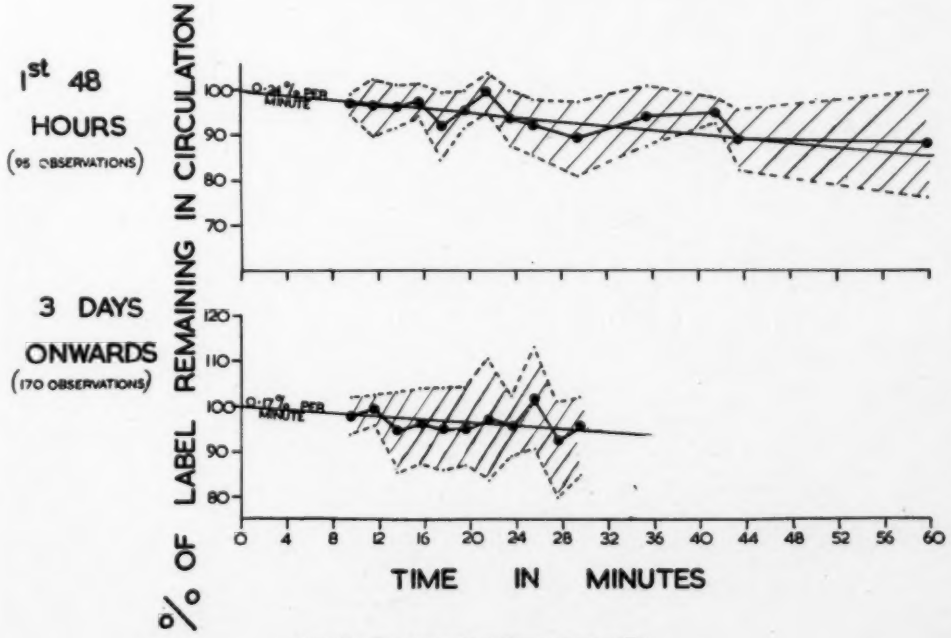

FIG. 1b.—The mean rate of disappearance of ^{32}P.

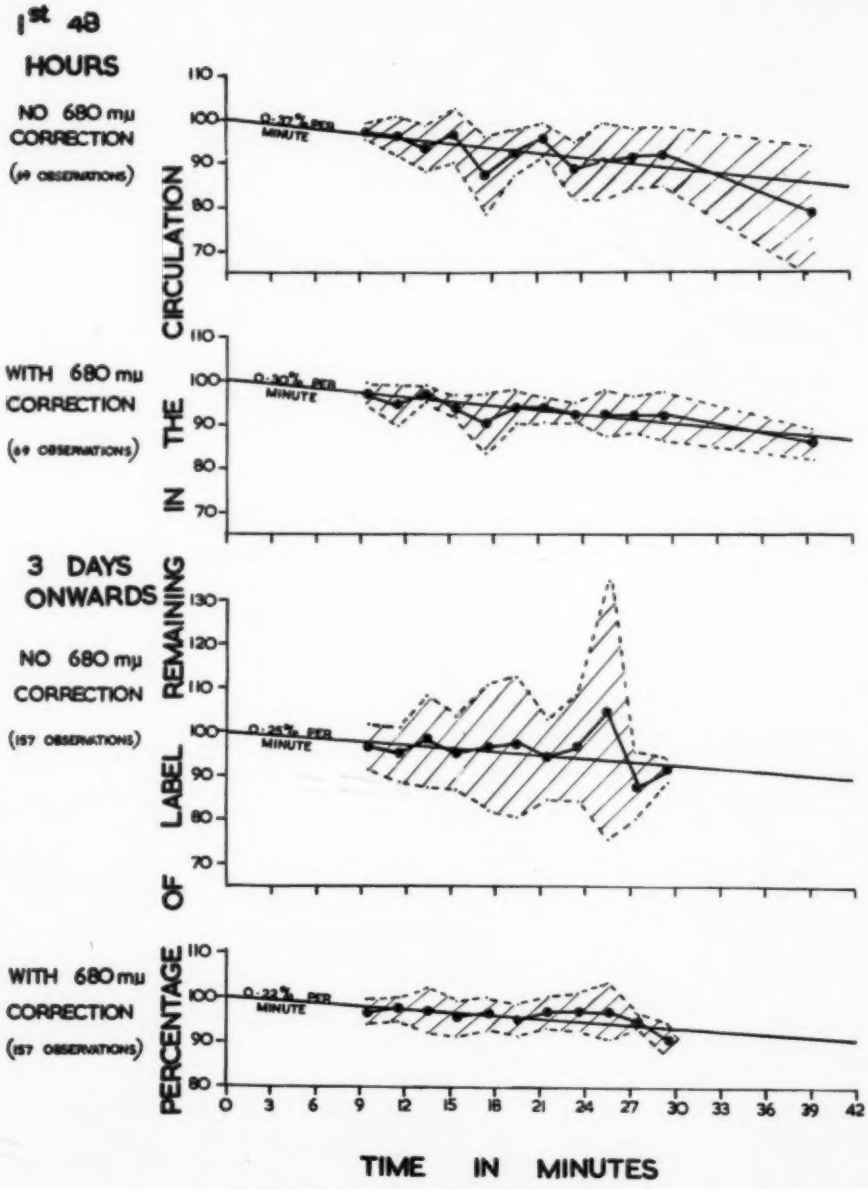

FIG. 1c.—The mean rate of disappearance of T-1824.

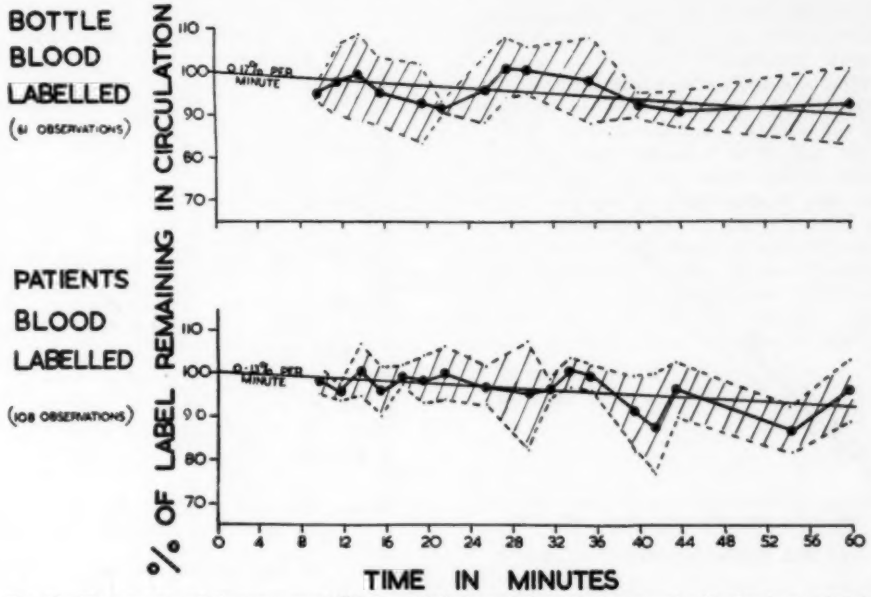

FIG. 1d.—The mean rate of disappearance of ^{51}Cr from labelled transfusion blood compared with that from labelled patient's blood.

TABLE III

NUMBER OF OBSERVATIONS EXCEEDING ±15% OF MEAN VALUES ILLUSTRATED IN FIG. 3 AND POSSIBLE SAMPLING ERRORS AFTER INJECTION OF LABELLED MATERIALS

Labelling Agent		9–14 min.	15–20 min.	21–30 min.	31–40 min.	Totals
^{51}Cr	Before 48 hours	0/28	2/36	2/27	2/24	6/115
	After 48 hours	0/8	0/10	2/12	0/7	2/37
^{32}P	Before 48 hours	0/22	0/32	0/24	0/7	0/85
	After 48 hours	1/57	5/70	8/41	—	14/168
^{51}Cr	Patient's cells labelled	0/27	0/28	2/21	1/20	3/96
	Stored cells labelled	0/9	2/18	2/18	2/11	6/56
T-1824 with 680 mμ correction	Before 48 hours	0/20	0/24	0/22	—	0/66
	After 48 hours	0/54	0/67	0/36	—	0/157
T-1824 without 680 mμ correction	Before 48 hours	0/20	0/24	0/22	—	0/66
	After 48 hours	3/54	9/67	7/36	—	19/157

the correction 19 out of 213 results exceeded these limits. The difference between the variances is highly significant ($P=0.001$).

Variations in Ratio between Body and Venous Haematocrits.—Ninety-six simultaneous estimations of red cell and plasma volume using either ^{32}P or ^{51}Cr and T-1824 enabled the body haematocrit to be calculated and hence its ratio to the venous haematocrit. The ratios (Table IV) have been considered at four periods after injury: the first (up to six hours after injury) when the circulation is most likely to be abnormal, the second (seven to 48 hours) during the post-operative period, the third (three to 30 days) when anaemia may be present in some patients, and finally (one to 12 months) when the circulation has probably returned to a normal condition. The mean ratio was 0.915 during the first 48 hours after injury compared with 0.948 later, but the scatter of the results is such that this difference is not significant ($\sigma=0.0641$, $t=2.04$, $P=0.05$). There is also no significant difference between the first and second periods ($\sigma=0.0583$, $t=1.602$, $P=0.10$). There was no greater range of variation during the first 48 hours than later, and the total range of all the results was ±13%. Only six of the 96 observations lay outside ±10%, and only 30 of the 96 outside ±5% of the mean value of 0.937. During the first six hours after injury the ratios did not exceed ±9% of the mean value.

Simultaneous Estimations of Red Cell and Plasma Volume.—These estimations were made by

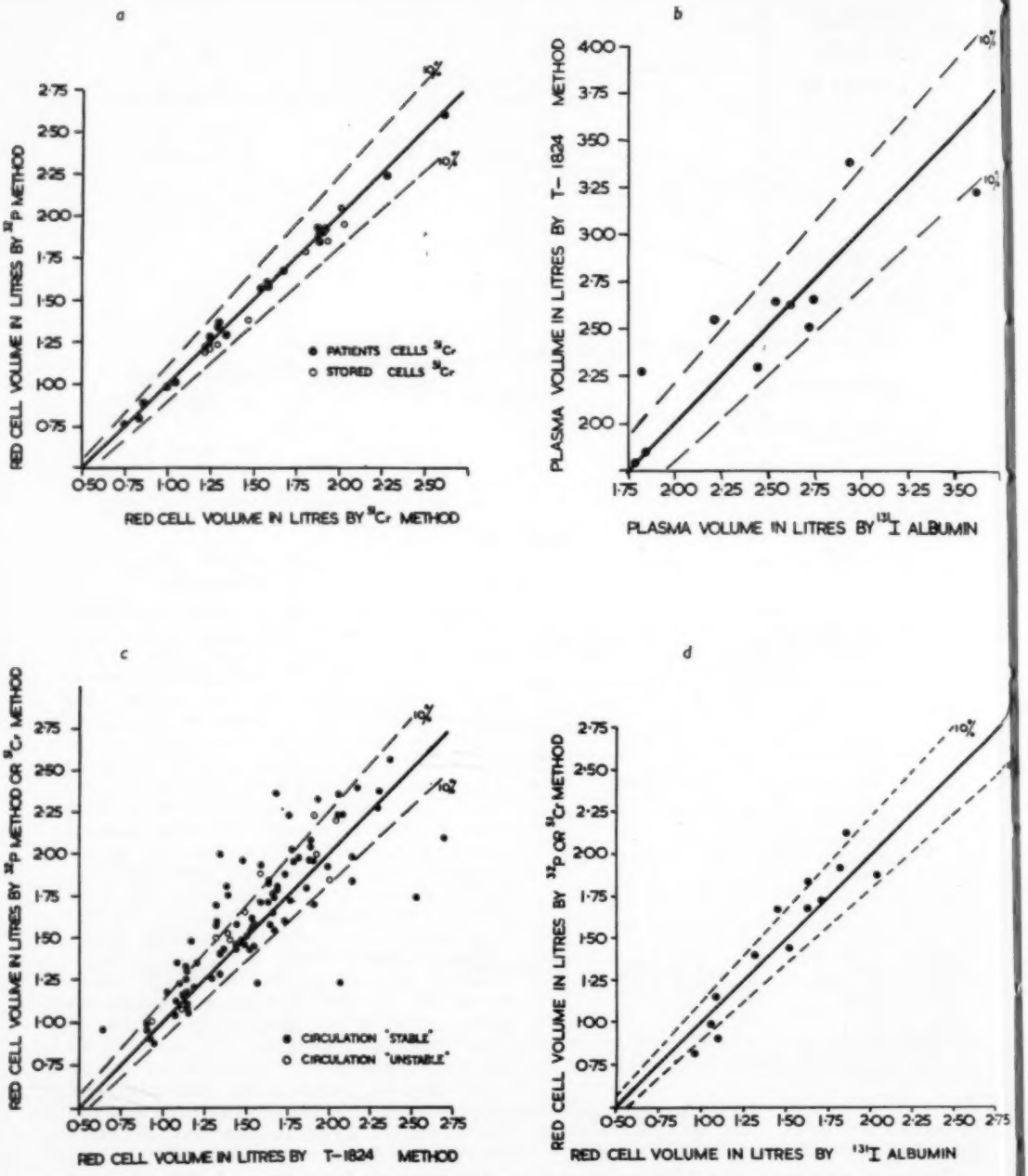

FIG. 2.—Simultaneous estimations of red cell and plasma volume using a number of methods. The scatter of the results around the line of equivalence is shown together with the ±10% limits.

TABLE IV
BODY/VENOUS HAEMATOCRIT RATIOS

First Six Hours after Injury	7–48 Hours	3–30 Days	1–12 Months	
0·97	0·96	0·93	0·89	0·95
0·85	0·77	1·00	0·96	0·89
0·97	0·82	1·06	1·00	0·99
0·92	0·92	1·00	0·88	0·90
0·95	0·88	1·02	1·00	0·86
1·00	0·95	0·97	0·92	0·90
0·97	0·94	0·91	0·99	0·89
0·88	0·89	0·87	0·96	1·02
0·95	0·77	0·85	0·93	0·90
0·94	0·99	0·90	0·97	0·95
0·89	0·91	0·84	0·94	0·95
0·94	0·88	0·88	0·99	0·96
0·93	1·03	0·91	0·92	0·93
	0·94	0·89	0·90	0·89
12·16	0·85	0·95	0·92	0·97
	0·91	0·94	0·94	0·95
n=13	0·91	1·24	0·93	1·03
	0·90	0·93	1·01	0·97
$\bar{x}$=0·935		0·93	0·92	0·96
	16·22	0·98	0·94	1·18
		0·90		0·95
	n=18	0·96		
		0·94		38·90
	$\bar{x}$=0·901	0·90		
				n=41
		22·70		
				$\bar{x}$=0·949
		n=24		
		$\bar{x}$=0·946		

two methods in parallel on the same subjects. The labelled materials were injected from two different syringes with not more than one-minute intervals between the injections. The amount of the two labels was determined separately in the sample of blood taken after mixing was complete. The results have been plotted to show their variance from the line of complete equivalence.

Red Cells from Patient Labelled with Both ^{32}P and ^{51}Cr.—The 19 estimations in this group are shown as solid circles in Fig. 2a. Fourteen of the 19 estimations were made within 24 hours of injury. All agree within a range of ±5% of the line of equivalence (S.D.=2.76, σ=0.63, P=0.5–0.6).

Red Cells from Patient Labelled with ^{32}P and Red Cells from Transfusion Bottle Labelled with ^{51}Cr.—The 11 estimations (hollow circles Fig. 2a) were made within 24 hours of injury. The red cell volumes determined with ^{51}Cr-labelled stored cells are slightly greater than those with ^{32}P-labelled patients' cells. All the results lie within a range of +9% to −1% of the line of equivalence. Older cells tend to give higher estimations (Fig. 3). The regression line was fitted from y= −1.147 + 0.426x. Only a small error results from using stored cells up to 10 days old.

Simultaneous Estimations of Red Cell and Plasma Volume Using ^{32}P- or ^{51}Cr-labelled Red Cells and T-1824 or ^{131}I-labelled Albumin.—Although the labelled red cells and albumin were usually injected with an interval between them of 15 to 20 minutes, on one occasion the interval was only one minute. With the sequential estimations the red cells were injected before the labelled albumin, so that mixing and sampling for the measurement of the red cell volume occurred before the plasma volume estimation.

Red cells labelled with ^{32}P and T-1824 were used on 90 occasions to estimate simultaneously the red cell and plasma volume. All the estimations were made at least 24 hours after injury when the circulation was thought to be "stable" (Fig. 2c). When the red cell volume calculated from the plasma volume and the haematocrit were compared with the directly estimated red cell volume, the two methods differed by amounts considerably greater than those found with the simultaneous ^{32}P and ^{51}Cr estimations. Inspection of the data shows that around the line of equivalence one-third of the observations agree to within ±5%; two-thirds of the observations agree to within ±10%; three-quarters of the observations agree to within ±15%; five-sixths of the observations agree to within ±20%. The red cell volume calculated from the plasma volume and the haematocrit was on average lower than that directly estimated.

The scatter of the results was no greater when the simultaneous estimations were made within four hours of injury.

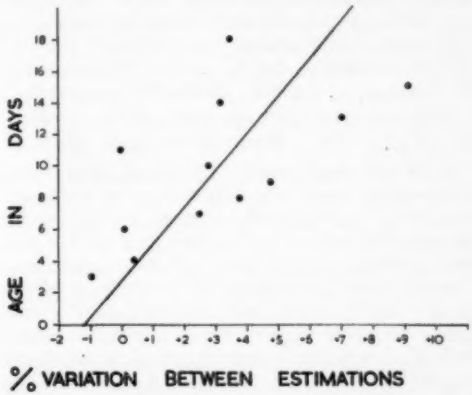

FIG. 3.—The relationship between the period of storage of red cells in A.C.D. solution before labelling with ^{51}Cr and the difference between simultaneous red cell volume estimations using ^{32}P labelled patient's cells and ^{51}Cr labelled stored cells.

The 13 simultaneous estimations using ^{131}I-labelled albumin and ^{51}Cr-labelled red cells also showed a considerable scatter (Fig. 2d).

Simultaneous Plasma Volume Estimations Using T-1824 and ^{131}I-labelled Human Albumin.—The two labelled materials injected within one minute of each other gave results (Fig. 2b) which agreed to within ±20% and seven of the 11 agreed to within ±10%. Only three of the estimations were made later than 48 hours after injury.

Discussion

This study has revealed variations in the amount of radioactivity in the suspending plasma saline, variations in the mean rate of disappearance of the labelled materials from the circulation, and differences between two methods used in parallel. These variations will be compared with the findings in normal individuals and discussed in terms of their probable effect on the accuracy of red cell volume measurement after injury.

Level of Radioactivity in Supernatant.—This indicates the amount of extracellular radioactivity found in the red cell suspension before its intravenous injection. Theoretically if the cells lose no more radioactivity later a supernatant level of 1% should produce a 1% error in the result. In practice, however, Chaplin (1954) found a 10% error in the red cell volume estimation when the supernatant level was only slightly more than 1%. We have found that when cells stored in A.C.D. solution were labelled with ^{32}P on average 3.3% of the radioactivity was in the supernatant. These high supernatant values were associated with very gross errors in the red cell volume when the latter was compared with a simultaneous ^{51}Cr.

Although supernatant values above 1% were occasionally found with both isotopes using the routine methods described above, there was no significant difference between the values determined within 48 hours of injury compared with those made later. The mean activity for ^{32}P was 0.57%, and is 0.33% higher than that reported by Chaplin (1954). This high mean value may have been due to the occasions when the labelled red cell suspension was left standing on the bench for up to half an hour before separation. In Chaplin's original method the suspension was centrifuged immediately. These results suggested that it is useful to test routinely the radioactivity level of the supernatant separated immediately at the end of preparation of the red cell suspension, and to review the red cell volume result with suspicion when the supernatant contains more than 1% of the total radioactivity.

The more detailed investigation of the rate of leakage *in vitro* of ^{51}Cr discussed above suggests that ^{51}Cr-labelled stored red cells may be kept for up to four hours before being injected. This is a useful advantage over ^{32}P as a red cell label, as the need for immediate injection is removed.

Adequacy of Intravascular Mixing and Survival of Labelled Materials in the Circulation.—The concentration of label per unit volume of blood or plasma at various times after injection has shown whether mixing is complete and also defined the survival of the material in the circulation. It is important that the sample be taken at a time when mixing is virtually complete, and yet before significant loss of the injected material from the circulation has occurred. Noble and Gregersen (1946) found the average mixing time of 27 normal males to be nine minutes, of 40 patients not in "shock" to be seven minutes, and of 27 patients in "shock" to be 14 minutes. In our patients the small scatter of the results nine to 15 minutes after injection suggests that mixing was complete by this time.

In conditions where the circulation may be abnormal samples may be taken 10, 15, 20, and 30 minutes after injection to look for unsuspected fluctuations in the concentration of labelled material. The theoretical possibilities of abnormal mixing after injury and the risks of failing to detect such abnormalities by repeated venous sampling have been discussed elsewhere (Topley *et al.*, 1959).

The changes in concentration of the label later than 15 minutes after injection have been used to show the rate of disappearance, although continued poor mixing could not be excluded. The standard deviations show no difference between the scatter of the results during the first 48 hours compared with later. In each case the mean rate of disappearance during the first 48 hours was slightly greater than that found later, and all values are slightly greater than those reported by other workers. The mean rates of disappearance later than 48 hours after injury are slightly greater than those of Mollison (1956) for T-1824 in normal individuals, and those of Chaplin (1954) for ^{32}P-labelled red cells. The recent work of Mollison (1956) also with ^{32}P-labelled red cells is more in agreement with our findings. Although the rate of disappearance of ^{51}Cr has been reported as negligible (Mollison and Veall, 1955), we find it to be 0.17% per minute for

observations before 48 hours after injury and 0.07% per minute for observations made later.

These rates of disappearance for the various labelled materials are very slightly greater than those used in the calculation of the red cell and plasma volumes published by Topley *et al.* (1959). Our evidence following injury supports the conclusions of Mollison (1956) and Wiklander (1956) on normal individuals that a normal average rate of disappearance of the labelled agent should be assumed rather than construction of a disappearance curve based on only two or three venous samples.

Assuming an average rate of disappearance, when and how often should venous samples be taken? Chaplin (1954) found slightly closer agreement between repeated red cell volume estimations in normal individuals when the results were calculated from two or more samples than when only one sample was used. The observations described above on the disappearance of the labelled material indicate that some results differed from the mean line by more than ±15%, and hence a red cell volume estimation may occasionally have been inaccurate by this amount. The smaller number of discrepancies nine to 15 minutes after injection suggests that a sample taken at this time may give a more accurate result than a sample taken later. A result based upon a single blood sample has the added advantage of simplification of the method leading to a quicker result. It is often advantageous, however, to take one or two samples during the ensuing half-hour to show any gross abnormalities and to accumulate evidence on the mixing time and rate of disappearance in the condition under investigation.

There is still very little information on the mixing time and the rate of disappearance of labelled materials in those patients with very severe, often fatal injuries, and more data are required on the validity of red cell volume estimations in these patients.

Ratio Between Body and Venous Haematocrits.—The concept of the body haematocrit and the possible reasons for it being about 90% of the venous haematocrit has been much discussed (Chaplin, Mollison, and Vetter, 1953; Mollison, 1956). The ratio is certainly affected by a number of different factors including errors in the red cell and plasma volume techniques. The close agreement between two red cell volume methods in parallel and the wider the scatter between two plasma volume estimations suggest a smaller error from the red cell volume techniques compared with the plasma volume methods. The ratio between the body and venous haematocrits did not show any greater variation during the first 48 hours after injury than later, but at all times variations did occur that could produce errors of more than ±10% in a red cell volume calculated from a plasma volume and the haematocrit.

Reeve and Veall (1949), Berson and Yalow (1952), Chaplin *et al.* (1953), and Brady, Cooper, Colodzin, McClenathan, King, and Williams (1953) have reported a smaller range of ratios between the body and venous haematocrits in normal individuals than in our patients. Nachman, James, Moore, and Evans (1950) have reported a range of 0.76 to 1.02 (mean=0.88) in 38 surgical patients, and Wasserman, Yoh, and Rashkoff (1951) have described a similar range in normal individuals. These results have been reviewed by Wiklander (1956).

Methods in Parallel and Series.—Many workers, including Mollison and Veall (1955), have shown good agreement (±5% error) between ^{32}P and ^{51}Cr red cell volume estimations in parallel, and we have confirmed this finding at varying times after injury. Chaplin (1954) has shown similar agreement when ^{32}P red cell volume estimations were repeated at weekly intervals. Reilly, French, Lau, Scott, and White (1954) have shown sequential red cell volume estimations two hours apart using ^{51}Cr to have a scatter of −4.0 to +10%.

A clinical assessment described by Topley *et al.* (1959) suggests that the red cell volume methods in series following many common forms of injury are usually consistent to within ±10%.

Simultaneous plasma volume estimations using T-1824 and ^{131}I-labelled albumin did not agree as closely as simultaneous red cell volume estimations. This result agrees with those of Crispell, Porter, and Nieset (1950), Brady *et al.* (1953), and Inkley, Brooks, and Krieger (1955), where approximately three-quarters of the results agreed to within ±10%. Aust, Chou, Marvin, Brackney, and Moore (1951) and Zipf, Webber, and Grove (1955) report a greater scatter. Which of the methods caused this scatter is unknown, although serial estimations using T-1824 and ^{131}I-labelled albumin by Crispell *et al.* (1950) suggest that the ^{131}I method is not much more accurate than the T-1824 method.

The results discussed so far suggest that each of the following methods has a place in the assessment of haemorrhage and oligaemia following injury.

The ^{32}P Method.—The published technique of Chaplin (1954) has been modified in minor detail as described above. Mollison, Robinson, and Hunter (1958) have since modified the method so that only two hours are required for an estimation instead of three. Two reports have appeared indicating the dangers of particulate matter in the stock solution of ^{32}P (Harrison and Raymond, 1951 ; Lamerton and Harriss, 1951). The material supplied by the Radiochemical Centre, Amersham, is satisfactory in this respect and the precautions described above, including sterilization within 20 minutes of opening, prevents contamination with micro-organisms. The radiation dosage associated with the use of ^{32}P will be discussed below.

The ^{51}Cr Method.—The method of Mollison and Veall (1955) is well validated for labelling the patient's own cells. Much less information is available on the accuracy of the method when stored cells are used. Theoretically red cells stored in A.C.D. solution and then labelled with ^{51}Cr may produce false red cell volume estimations due to the disappearance of a significant number of labelled non-viable cells from the circulation within 15 minutes of intravenous injection. As these cells would be absent from the blood sample taken for the estimation the result will be falsely high. However, as described above, estimations in parallel with ^{32}P showed good agreement. There was no significant difference in the rate of disappearance of ^{51}Cr from the circulation when labelled stored cells were compared with labelled patients' cells. The experiments describing the low rate of leakage at room temperature of ^{51}Cr from labelled stored cells indicate that these cells can be kept for at least three hours before the test is performed. This is an advantage over ^{32}P-labelled red cells, particularly when the laboratory is some distance from the ward.

It would therefore seem that ^{51}Cr-labelled stored red cells less than 10 days old are clinically useful for estimating red cell volume. Meyer (1956) has suggested using Group O Rh-negative red cells labelled with ^{51}Cr. We have found stored cells of the appropriate ABO and Rh group of particular advantage in an emergency when venous sampling may be difficult due to oligaemic "shock."

The T-1824 Method.—The method of Reeve (1948) has been modified as described above by applying a correction for variations in optical density not due to T-1824. This correction is particularly necessary in the hours immediately following injury, as lipaemia is then often present. Similar corrections for opacity have been recommended by Shapiro (1951) and Hamilton (1958). Gregersen (1951) shows the advantage of using a simple photo-electric instrument and has stressed the optical disadvantages of using a delicate spectrophotometer to measure opaque solutions. In practice, however, our simple method using a spectrophotometer has shown significantly less variation in the rate of disappearance of the dye compared with that when no opacity correction is made.

Most of the extraction methods, e.g., those of Allen, Ochoa, Roth, and Gregersen (1953) and of Hobsley and Dew (1958), are not much more accurate than careful direct methods using a correction of opacity and are more time consuming. The method described by Tornberg (1958) may be simple enough for emergency use, but it is doubtful whether its accuracy is any better that a direct method allowing for varying plasma opacity.

Repeated T-1824 plasma volume estimations are not completely without risk, due mainly to the possible effect of dye bound to living cells. Roberts (1954) reports a fatality from septicaemia following an injection of T-1824, but there is no evidence that the plasma volume estimation was responsible. Cells of the reticulo-endothelial system readily engulf the dye, and Hueper and Ichniowski (1944) report adverse effects on liver, heart, and lung cells of dogs, cats, rabbits, and rats. Allen and Orahovats (1951) have shown a decreased oxygen consumption by liver slices exposed to T-1824. Although dog red cells have been shown by Allen and Gregersen (1953) to be lysed by 16–28 mg. of free dye, this concentration does not affect thrice washed human red cells. The dye bound to albumin had no effect on the red cells of any species. The blue colour of the skin caused by quantities of dye in excess of those usually given for a plasma volume estimation lasts a considerable time. The concentration of dye recommended by Gibson and Gregersen (1935) of 0.15 to 0.20 mg. per kg. for a single plasma volume estimation appears to be without ill effects and is very much less than the amounts producing the harmful effects discussed above.

The advantage of the T-1824 method over that using ^{131}I-labelled albumin is the absence of any radiation hazard. For the estimation of red cell volume both plasma volume methods have the disadvantage of being less accurate due to variations in the ratio between the body and venous haematocrits. The T-1824 method has

the added disadvantage of requiring a large blood sample (15 ml.) before the injection of the dye, which is sometimes not available in the presence of oligaemic "shock."

The ^{131}I Method.—This method has been used as the simple quick "blood volume" guide, as it takes less laboratory time than any other method. All the evidence to date suggests that it is at least as accurate as the T-1824 method.

Radiation Dosages.—A limiting factor in the use of radioactive isotopes is the potential danger arising from the radiation emitted during their disintegration. Reeve and Veall (1949) have calculated that 1 μC of ^{32}P per gram of tissue delivers a dosage of 875 r.e.p. during its complete radioactive life. Using these figures, the small dose of ^{32}P used for a red cell volume estimation of 2 μC generally distributed in a 70-kg. adult gives a total dose of 0.025 r.e.p. This figure is based on the assumption of no excretion, but it is only a theoretical value as fairly rapid excretion does occur.

With ^{51}Cr, Frank and Gray (1953) have calculated, using the formula of Marinelli, Quimby, and Hine (1948), that 25 μC gives a total dose of 0.025 r.e.p. In the calculation it was assumed that no excretion occurred, that the γ radiation irradiated the whole body, and that the very soft x rays irradiated the blood and reticulo-endothelial cells only.

The relatively rapid loss of ^{32}P from the circulation makes possible repeated estimations, e.g., daily, without an increase in the amount of radioactivity required for the test. This is in contrast to ^{51}Cr, where the persistence of the label in the red cells makes it necessary to use twice the amount of activity for an estimation as was used for the previous estimation. The advantage that ^{32}P has in this respect is offset in emergency work because three hours are required for the determination compared with one and three-quarters to two hours with ^{51}Cr. The modification of the ^{32}P method by Mollison *et al.* (1958) should reduce this disadvantage.

A further reduction in radiation dosage to 0.009 r.e.p. is obtained by the estimation of plasma volume instead of red cell volume with ^{131}I-labelled albumin.

The radiation dosages described above are less than administered for a distant chest radiograph, where average doses between 0.04 and 0.2 r.e.p. are used (Recommendations of the International Commission on Radiological Protection, 1954).

Risks of Contamination of Labelled Material.—There is a theoretical risk of bacterial contamination of the blood during the labelling procedure, and thus the possibility of a blood stream infection following its intravenous injection. Such risks might be increased if the research procedure of blood volume estimations became a routine. There has been no clinical evidence of such an infection from contaminated blood in the 1,000 tests performed in this laboratory. Reeve (1950) laid great emphasis on the need for reinjecting the blood within two hours of sampling to avoid gross bacterial contamination.

The greatest risk may be contamination of the stock solution of radioactive material. Such a risk can be reduced to negligible proportions by effective sterilization (M.R.C. Report, 1959) immediately after using the stock solution. A small sterilizer or pressure cooker in the same laboratory as that used for blood volume estimations ensures no delay in sterilization. The sterilizer can be checked by repeated temperature recordings during sterilization, by occasional checks with bacterial spores of known resistance to heat, and by the invariable use of "Browns" tubes (Kelsey, 1958; Lowbury, 1958).

Summary

Red cell volume estimations have been made at various times after injury using ^{32}P- and ^{51}Cr-labelled red cells, and plasma volumes using either T-1824- or ^{131}I-labelled albumin.

A survey of the amount of extracellular radioactivity, the disappearance curves of labelled materials from the circulation, the ratios between the body and venous haematocrits, and a combination of different methods in parallel led to the following conclusions:

(*a*) That there was no clinically significant difference in the technical accuracy during the first 48 hours after injury compared with later.

(*b*) That most of the results were not in error by more than $\pm 10\%$.

(*c*) That direct red cell volume methods using ^{32}P and ^{51}Cr were considerably more accurate measures of red cell volume than the indirect method using a plasma volume and the haematocrit, and were therefore considerably more precise in the assessment of haemorrhage.

The technical details of routine red cell and plasma volume estimations after injury are discussed.

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A NEW ADSORPTION AGENT FOR COAGULATION FACTORS

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Various bentonites constitute about 90% of montmorillonite, an ash of volcanic origin. The bentonites are essentially hydrated aluminium silicate crystallized into fine laminae, and are used in industry for filtration and clarification. We have been unable to find any reference in the literature to the use of bentonite in the adsorption of coagulation factors.

Techniques

"Prolabo" bentonite is a colourless powder, the composition of which is set out in Table I. The adsorption of plasma or serum was carried out at

TABLE I

COMPOSITION OF BENTONITE

Ash	7·70 %
Silica	58·30 %
Aluminium	19·0 %
Iron oxide	5·10 %
Calcium	2·60 %
Magnesium	1·70 %
Alkali, excluding TiO_2	5·60 %
Density	0·70

room temperature for exactly 10 minutes with constant gentle shaking. The methods for the assay of coagulation factors have been described elsewhere (Soulier and Larrieu, 1958).

Results

The adsorption of coagulation factors was studied in the presence of the following decalcifying agents: Ion exchange resins, ammonium citrate, ammonium oxalate, a mixture of potassium and ammonium oxalate, and sodium E.D.T.A. Whichever decalcifying technique was used, the results were similar and only the results using human citrated plasma (1 part of citrate 0.38% to 9 parts of blood) are reported here. Citrated plasma was exposed to different concentrations of bentonite and the concentration of the various coagulation factors was measured thereafter. The results of these various assays are reported in Table II. When a concentration of bentonite higher than 0.5% was used, it will be seen that the clotting time on recalcification and the Quick prothrombin time were all infinitely long. This suggests that fibrinogen had been adsorbed, a conclusion which was confirmed by the following

TABLE II

ABSORPTION BY BENTONITE FROM CITRATED PLASMA

	Control Clotting Time of Test without Added Plasma (Sec.)	Concentration of Bentonite per 1,000 (w/v)						
		0	0·1	0·5	1	2	5	10
Recalcification		95	65	115	+900	—	—	—
Quick		11	10	20	+900	—	—	—
Pro-accelerin	150	23	23	30	72	148	154	154
Proconvertin	90	19	17	21	41	—	—	—
Prothrombin	96	15	14	14	15	23	97	97
Thrombin		15	15	73	+900	—	—	—
A.H.G.	435	200	200	225	350	435	430	435
Christmas factor	660	210	200	200	310	—	—	—
Hageman factor	390	195	150	195	230	330	375	400
P.T.A.	750	305	270	360	450	600	700	700

observations: no precipitate occurred on heating this plasma to 56° C. for five minutes and electrophoresis of the adsorbed plasma showed no spot between the β and γ globulins. It will also be seen that antihaemophilic globulin (VIII) and pro-accelerin (V) were adsorbed when a concentration of about 1% bentonite was used. The factor VII complex (proconvertin and Stuart factor) and Christmas factor (IX) were adsorbed at a concentration of 2%, while at this concentration only about 30 to 40% of true prothrombin was adsorbed. Finally, Hageman factor and P.T.A. were only adsorbed when 5% bentonite was used.

Other experiments not reported here substantiated the following points: (1) The adsorption of the various factors is equally satisfactory in the presence of citrate and oxalate and E.D.T.A. Adsorption is adequate in the absence of an anticoagulant (resin-treated plasma and serum). (2) The order and degree of adsorption of the different coagulation factors by bentonite

were constant for the same lot and for two different lots of bentonite obtained from the same firm. (3) Two successive adsorptions by 1% bentonite are little more efficacious than one exposure to 2% bentonite. (4) Heparin co-factor, antithrombin, and heparin were not adsorbed by the bentonite between the concentrations of 0.5 and 5%. Plasminogen was more easily adsorbed than antiplasmin. The ionized calcium of plasma or serum was not adsorbed by 2% of the calcium salt of bentonite.

Applications

There are many uses for the adsorption technique with bentonite in the coagulation laboratory. In Fig. 1 the adsorptive power of bentonite on the various coagulation factors is represented diagrammatically, and reference to this will show how it is possible to separate fibrinogen from antihaemophilic factor and how prothrombin may be purified.

Separation of Fibrinogen and Antihaemophilic Globulin.—By using citrated or oxalate plasma or Cohn fraction 1 (E.D.T.A. plasma destroys antihaemophilic globulin) and bentonite 0.5%, it is possible to obtain antihaemophilic globulin separated from fibrinogen. Results of this differential adsorption are illustrated in Tables III and IV. It will be seen that bentonite is superior to Fuller's earth, since Wagner, Richardson, and

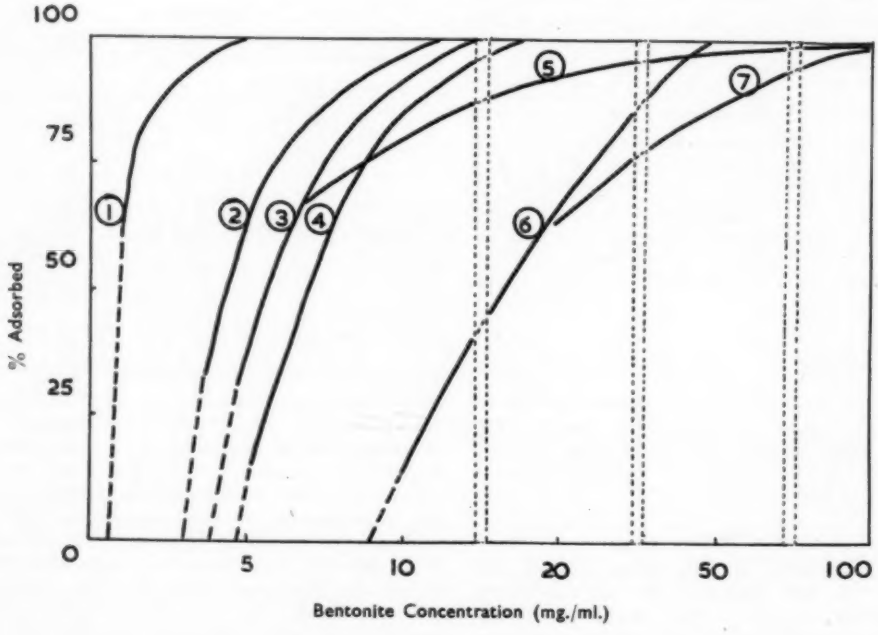

FIG. 1

TABLE III

SEPARATION OF A.H.G. FROM FIBRINOGEN IN CITRATED PLASMA

Bentonite (mg./ml.)	Fibrinogen		Antihaemophilic Globulin	
	Thrombin Clotting Time (Sec.)	Percentage of Normal	One-stage Estimation (Sec.)*	Percentage of Normal
0	14	100	215	100
1	17	30	215	100
3	22	18	215	100
4	40	12	220	60
5	+600	0–10	225	50
7		0	230	45
9		0	240	40

* Control time in absence of A.H.G.: 600 sec.

TABLE IV

SEPARATION BY BENTONITE OF A.H.G. IN COHN'S FRACTION I (PROTEIN CONCENTRATION 0·4%) FROM FIBRINOGEN

Bentonite (mg./ml.)	Fibrinogen		Antihaemophilic Globulin	
	Thrombin Clotting Time (Sec.)	Percentage Remaining	One-stage Estimation (Sec.)*	Percentage Remaining
0	14	100	235	100
3	50	11	235	100
4	+200	0	305	28
5	+200	0	315	22
6	+200	0	450	7

* Control time in absence of A.H.G. = 540 sec.

Brinkhous (1957) claim that 20 times more Fuller's earth is required to adsorb human fibrinogen. Bentonite was used at the same concentration as aluminium hydroxide, but has the advantage that it does not have to be prepared specially and that the results are highly reproducible. Using bentonite we have been able to prepare a sample of human antihaemophilic globulin, free of fibrinogen, which was 70 times more active than fresh human plasma as judged by the weight of protein.

Purification of Prothrombin.—Bentonite used at a concentration of 2% produced the following effects: Fibrinogen, antihaemophilic globulin, Christmas factor, and proconvertin were removed, while prothrombin and a part of factors P.T.A. and Hageman were left. To isolate prothrombin treated plasma was further adsorbed selectively with tricalcium phosphate (Baker) 0.5%.* We have been able to obtain several samples of purified prothrombin by this method which contained trace quantities of factor VII and Christmas factor too small to be measured: even the prothrombin prepared by Seegers (1952) is not completely free of these two factors. Table V records an example of some actual results obtained. By this method it is possible reproducibly to obtain purified prothrombin concentrated (600 units/mg.) and with a high yield. There seems to be no reason why a higher concentration should not be obtained. Electrophoresis on starch block shows that this fraction produces a single band in the α_2 globulin position.

*P. Didisheim, working in this laboratory, has shown that Baker's tricalcium phosphate is by far the most active of all the adsorbents of the prothrombin complex and Christmas factor.

TABLE V

PURIFICATION OF PROTHROMBIN IN OXALATED (0.15%) PLASMA

	Prothrombin		Proconvertin Complex (VII)		Christmas Factor (XI)	
	Time (Sec.)	Percentage Activity	Time (Sec.)	Percentage Activity	Time (Sec.)	Percentage Activity
Control: Reagents only: ..	138	0	480	0	1,200	0
Unabsorbed plasma	14	100	18	100	260	100
Plasma absorbed with bentonite, 20 mg./ml.	16	48	182	0–1	876	0–1
Plasma absorbed with tricalcium phosphate, 5 mg./ml.	128	0–1	480	0	1,200	0
Eluate in ½ plasma volume of 0·18 M trisodium citrate, dialysed	15	75	120	0–1	945	0–1
Euglobulins at *p*H 5·2 redissolved in 1/10 volume: 0·26% protein	11·5	300	105	0–1	900	0–1
	Two-stage estimation: 1,500 units					

Bentonite and the Contact Factor

The contacting activity of bentonite on normal citrated plasma, using the low concentration of 1 mg./ml. which does not adsorb coagulation factors, has been studied and compared with that of kaolin, which has been studied extensively by Margolis (1957). All tests were carried out in silicone-treated tubes and the results are

TABLE VI

ACTIVATION OF CITRATED PLASMA BY BENTONITE OR KAOLIN

	Clotting Time of Test without Added Plasma	Untreated Plasma (Sec.)	Plasma Exposed to 1 mg. Bentonite per ml. (Sec.)	Plasma Exposed to 1 mg. Kaolin per ml. (Sec.)
Recalcification		125	105	125
Quick ..		14	13	13
Pro-accelerin ..	75	30	28	27
Proconvertin ..	+240	29	25	28
Prothrombin ..	72	18	18	18
A.H.G. ..	+600	330	200	270
Christmas factor	+600	260	165	180
Hageman factor	525	330	195	200
P.T.A. ..	660	310	220	220

summarized in Table VI. It will be seen that bentonite apparently activates the contact factor to a greater degree than an equal weight of kaolin, and activation is shown by the shortening of the recalcification and Quick times. It diminishes the apparent concentration of factor VII, P.T.A., and Christmas factor, and it interferes to some extent with the estimation of antihaemophilic globulin. (These findings are reported in full in another paper devoted to a further study of the contact factor.)

Stabilization of Plasma by Heat

Citrated plasma treated with 5% bentonite, or with 0.5 to 1% in the presence of mandelate (a stabilizing anion used during heat treatment of the albumin fraction), will not gel when heated to 60° C. for several hours. Thus it is possible to use this simple procedure to stabilize plasma before the heat treatment required to destroy the virus of infective hepatitis. The more complex treatments proposed (Nitschmann, Kistler, Renfer, Hässig, and Joss, 1956) are then unnecessary.

Summary

Bentonite is an adsorbent of coagulation factors. It adsorbs fibrinogen at a concentration of 0.5% and fibrinogen can thus be removed from plasma or from plasma fractions containing antihaemophilic globulin. True prothrombin can be separated from the other coagulation factors normally present in prothrombin preparations. Bentonite 0.1% is an even greater activator of the "contact factor" than kaolin. Five per cent. bentonite prevents the gelling of plasma at 60° C.

We wish to thank Mlle. Weilland and Mme. Prou-Wartelle for their excellent technical assistance. We also wish to thank Dr. J. R. O'Brien for his kindness in translating this paper.

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LEUKAEMOID REACTIONS IN DISSEMINATED TUBERCULOSIS

BY

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Secondary haematological changes in tuberculosis may mimic many blood dyscrasias, including leukaemia. The appearance of primitive white cells in the peripheral blood is probably more common in disseminated than in pulmonary tuberculosis (Muller, 1943; O'Brien, 1954). The reaction may involve any cell type: myeloid reactions have been reported by Marzullo and deVeer (1931) and Custer and Crocker (1932), lymphatic reactions by Gardner and Mettier (1949) and Staffurth and Spencer (1950), and a monocytic reaction by Gibson (1946).

The term "leukaemoid reaction" was introduced by Krumbhaar (1926) to describe blood changes "indistinguishable from one of the various forms of leukaemia." In many cases a leukaemoid reaction can be distinguished from leukaemia by considering the clinical and laboratory findings. In some, especially those in which the leukaemoid reaction is secondary to disseminated tuberculosis, diagnosis is difficult or impossible and the true nature of the haematological changes is recognized only at post-mortem examination.

The two following cases presented with haematological changes suggestive of myeloid leukaemia. Disseminated "non-reactive" tuberculosis, characterized histologically by widespread foci of severe necrosis containing many tubercle bacilli and with little or no peripheral cellular response, was found at post-mortem examination.

Case Reports

Case 1.—A soldier, aged 19, was admitted to hospital complaining of cough and breathlessness on moderate exertion of four days' duration.

Clinical Findings.—The patient looked pale and ill. There was slight faucial injection. The temperature was 103° F. (39.4° C.). Signs of consolidation were present at the base of the left lung. Fine rales were audible over both lung apices.

Laboratory Findings.—The haemoglobin, estimated by the alkaline haematin method, was 9.9 g. per 100 ml.; the total white cell count was 4,400 cells per c.mm., and a differential count showed 31% of myelocytes and myeloblasts; the platelets were 73,000 per c.mm.; the E.S.R. was 29 mm. in one hour (Wintrobe). Smears of sternal marrow aspirate showed normoblastic erythropoiesis in a cellular marrow; the myelo-erythroid ratio was increased, with numerous myeloblasts and myelocytes; megakaryocytes and platelets were scanty. Chest radiographs showed left basal consolidation.

Course and Treatment.—Antibiotic treatment was given for 12 days. The patient improved subjectively. The temperature fell to normal and signs of consolidation at the left base diminished. Because the blood picture did not return to normal the patient was transferred to the Queen Alexandra Military Hospital for investigation 17 days after the onset of symptoms. Examination of histological sections (Cappell, Hutchison, and Smith, 1947) and smears of a second specimen of sternal marrow aspirate showed increased cellularity and a higher proportion of myeloid precursors (Fig. 1); no tubercles were seen. A diagnosis of acute myeloid leukaemia was made and treatment with A.C.T.H., 80 units daily, started. Throughout the course of the disease, there was pyrexia of 101–103° F. (38.3–39.4° C.) and a progressive fall in the haemoglobin to 8.7 g. per 100 ml. despite blood transfusions. The white cell count varied from 12,000 to 20,000 cells per c.mm. with a fall to 1,700 cells per c.mm. three days before death; on each occasion a differential count showed 46 to 56% of myelocytes and myeloblasts. The patient died in coma 120 days after the onset of symptoms.

Post-mortem Findings.—Scattered petechial haemorrhages were present in the skin and mucous membranes. Enlarged lymph nodes were present in the neck, axillae, groins, and both lung hila. There was confluent consolidation of both lung bases. The

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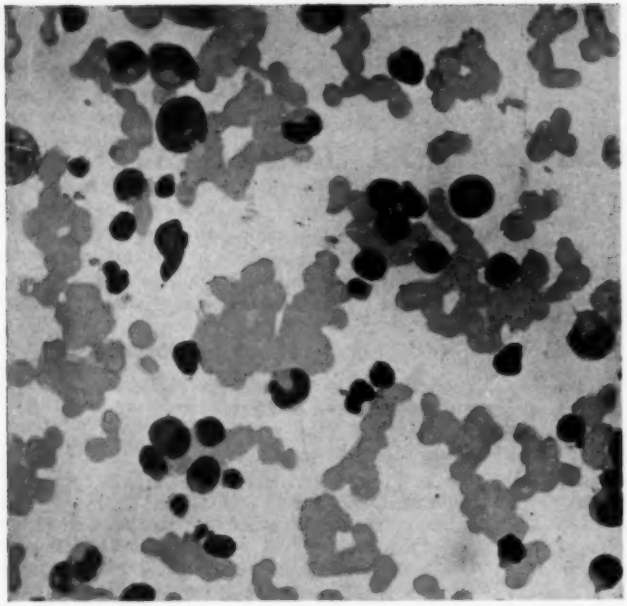

FIG. 1.—Bone marrow aspirate (Case 1) showing preponderance of myeloid series (Jenner-Giemsa, × 325).

white cell counts of 27,000 to 41,000 cells per c.mm.; 13% of myelocytes were present on each occasion. Smears of sternal marrow aspirate showed an increase in the myeloerythroid ratio due to increased numbers of myeloblasts and myelocytes. The patient was admitted to hospital for investigation.

Clinical Findings. — There were signs of loss of weight. The lower pole of the right kidney was palpable. Fine rales were heard throughout the chest.

Laboratory Findings. — A chest radiograph showed pleural thickening. Intravenous pyelography and a barium meal and "follow-through" were negative. No tubercle bacilli were isolated from, or malignant cells seen in, serial specimens of sputum. The haemoglobin was 12.1 g. per 100 ml.; the total white cell count was 33,000 cells per c.mm.; a differential count showed 16% of myeloblasts and myelocytes. A small amount of marrow was aspirated with difficulty

spleen (512 g.) was enlarged and the capsule adherent to the splenic flexure of the colon and the left lobe of the liver. Many white foci, 3 to 5 mm. in diameter, were present on the surface of the spleen and throughout the substance. The liver, gut, kidneys, pancreas, endocrine glands, sternum, and ribs were grossly normal. The brain and spinal cord were not examined.

Sections from both lungs, liver (Fig. 2), kidneys, suprarenals, lymph nodes, pancreas, spleen, and sternal marrow showed scattered foci of non-reactive tuberculosis. Sections stained by the Ziehl-Neelsen technique showed acid- and alcohol-fast bacilli within the foci. Organisms were more numerous in sections from the liver and spleen; none were seen in sections of sternal marrow. There was no leukaemic infiltration of the organs examined.

Case 2.—A man aged 50 complained at a routine medical examination of lassitude, loss of weight, and indefinite epigastric pain. Physical examination was negative. The total white cell count was found to be 34,000 cells per c.mm.; a differential count showed 19% of myelocytes. The E.S.R. was 26 mm. in one hour (Wintrobe). Blood examinations during the next month showed total

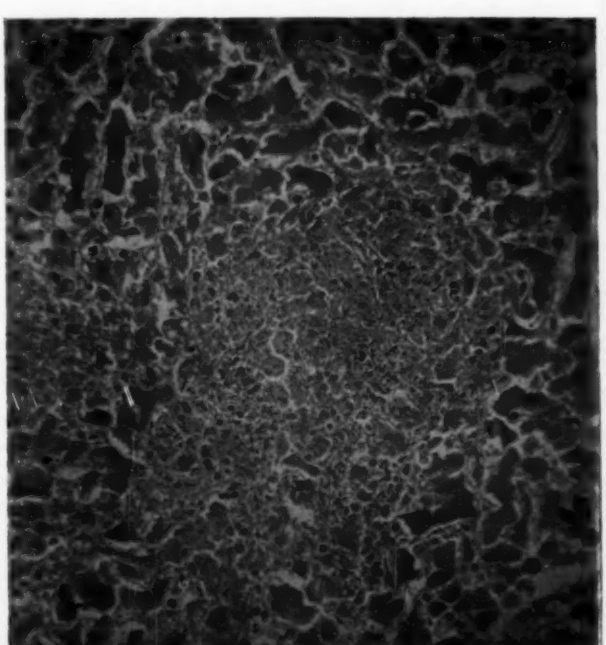

FIG. 2.—Liver (Case 1) showing a necrotic focus with minimal peripheral cellular reaction (haematoxylin and eosin, × 150).

from the iliac crest; smears showed an obvious increase in the number of primitive granular cells. These changes were thought to represent a leukaemoid reaction or early myeloid leukaemia. The appearances of the leucocytes in blood films and marrow smears stained for phosphatase by the method described by Leonard, Israëls, and Wilkinson (1958) supported a diagnosis of leukaemia.

Course and Treatment.—A blood transfusion was given and treatment with prednisolone, 20 mg. t.d.s., started. During the next 15 days the liver and spleen became palpable. The total white cell count varied from 14,000 to 23,000 cells per c.mm.; on each occasion a differential count showed 2 to 5% of myelocytes. The patient's condition deteriorated rapidly and he died in coma 55 days after admission to hospital.

Post-mortem Findings.—A fibrotic focus, 4 mm. in diameter, was present in the right lung apex. The hilar lymph nodes were enlarged. The spleen was enlarged; many white foci, 2 to 4 mm. in diameter, were present in the substance. The liver showed similar appearances. Enlarged lymph nodes were present in the porta hepatis and round the coeliac axis. The gut, pancreas, kidneys, heart, brain, and endocrine glands were grossly normal. The femur showed pinkish, gelatinous marrow extending to mid-shaft.

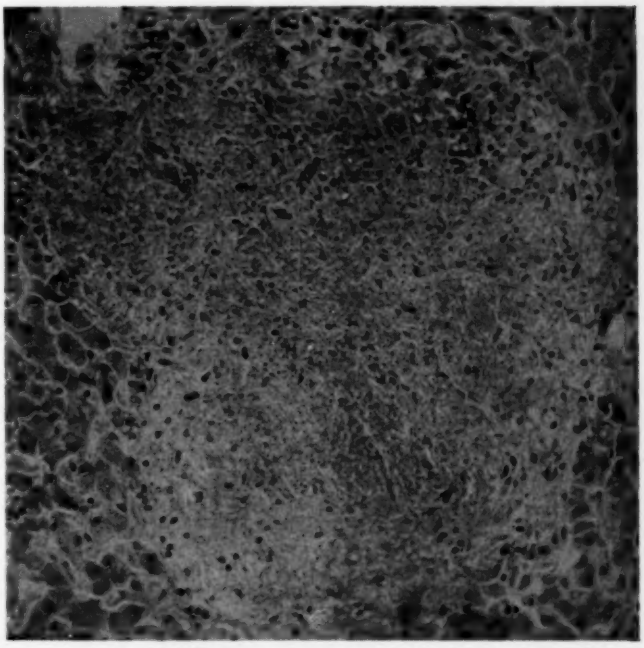

FIG. 3.—Liver (Case 2) showing a necrotic focus with minimal peripheral cellular reaction (haematoxylin and eosin, × 150).

Sections from the liver (Fig. 3), spleen, hilar and abdominal lymph nodes showed many foci of non-reactive tuberculosis in which large numbers of acid- and alcohol-fast bacilli could be demonstrated. The apex of the right lung showed a small nodule of acellular fibrous tissue; scanty acid- and alcohol-fast bacilli could be demonstrated in a small adjacent focus of non-reactive tuberculosis. No tubercles were seen in sections from other parts of the lungs. Sections of femoral marrow showed myeloid hyperplasia; small areas of necrosis, containing very scanty acid- and alcohol-fast bacilli, were present. There was no leukaemic infiltration of the organs examined.

Discussion

Two main difficulties arise in the diagnosis of such cases.

First, exclusion of disseminated non-reactive tuberculosis is difficult because the clinical picture, varying from a rapidly fatal, typhoid-like state to a chronic wasting illness lasting for several months, is ill defined and not specific (O'Brien, 1954), and ordinary examinations to exclude tuberculosis, e.g., chest radiography, examination of serial specimens of sputum, and examination of the choroid for tubercles, are often negative when the disease takes this form (Emery and Lorber, 1950; Fountain, 1954; Medd and Hayhoe, 1955).

Secondly, there is no wholly satisfactory way of differentiating the peripheral blood and bone marrow changes of leukaemia from those of a leukaemoid reaction. The total leucocyte count is of little or no diagnostic significance unless it is greater than 100,000 cells per c.mm. (Heck and Hall, 1939). Leonard *et al.* (1958) consider that the determination of the alkaline phosphatase in the leucocytes is at present the best, and in many cases the only, means of distinguishing between myeloid leukaemia and leukaemoid reactions involving the myeloid series of cells. The results using this technique were misleading in our second case.

If the ordinary methods used in the diagnosis of tuberculosis are negative, they should be

supplemented by examination of bone marrow and liver tissue obtained by needle biopsy.

Tubercles and tubercle bacilli have been demonstrated during life and at necropsy in the marrow of patients with disseminated tuberculosis. Schleicher (1946) demonstrated tubercles in specimens of marrow from eight patients during life. Emery and Gibbs (1954) aspirated 0.1 ml. amounts of sternal or rib marrow during post-mortem examination of 44 children who died from miliary tuberculosis and demonstrated tubercles in 14 of the specimens. They emphasize that had this examination been carried out during life it would have led to the correct diagnosis in five cases in which chest radiography and choroidal examination were negative. Tubercles in the marrow have also been demonstrated at necropsy by Ball, Joules, and Pagel (1951), Gardner and Mettier (1949), Friend and Thackray (1952), and Fountain (1954).

In addition to the examination of smears and histological sections of marrow for tubercles and tubercle bacilli, marrow should be cultured on Löwenstein-Jensen medium and injected into a guinea-pig (Schleicher, 1946; Ball *et al.*, 1951; Emery and Gibbs, 1954; Fountain, 1954; and Medd and Hayhoe, 1955).

The liver is always and often extensively involved in non-reactive tuberculosis according to O'Brien (1954). Mather, Dawson, and Hoyle (1955) found that needle biopsy of the liver gives histological evidence of tuberculosis more often in miliary tuberculosis than in other forms of the disease. Medd and Hayhoe (1955) diagnosed one case by this means and suggest that it could be used in the diagnosis of disseminated non-reactive tuberculosis.

If these investigations, and lymph node biopsy where possible, are negative, empirical treatment with anti-tuberculous drugs should be considered and is justified by the rapidly fatal course of disseminated tuberculosis if left untreated. Paulley (1954) has so treated one patient with apparent success. Medd and Hayhoe (1955) successfully treated one case diagnosed by liver biopsy. They suggest that anti-tuberculous drugs may have to be given for several weeks before any clinical improvement is noted and should be given for more than a year to prevent relapse. Because of the frequency with which cortisone and A.C.T.H. are used in the treatment of leukaemia, and the effect which these drugs may have on the course of a tuberculous infection, it is important to distinguish between leukaemia and leukaemoid reactions (Hughes and Thomson, 1957). Anti-tuberculous drugs should be given in those cases where the diagnosis is in doubt and treatment with steroids is considered.

There is little agreement about the significance of the leukaemoid reactions which accompany tuberculosis; they have been regarded on the one hand as a response of the bone marrow to tuberculous infection, and on the other as representing true leukaemia. The frequency with which such reactions occur in disseminated tuberculosis with demonstrable bone marrow involvement, the absence of characteristic leukaemic infiltration of organs examined at necropsy, and the occurrence of other blood dyscrasias in association with disseminated tuberculosis lead us to believe, with Marzullo and deVeer (1931), Heck and Hall (1939), Gardner and Mettier (1949), and others, that the haematological changes are secondary to tuberculous infection. Mönckeberg (1912), quoted by Marzullo and deVeer, suggests, however, that tuberculous infection may so alter leukaemia that the characteristic leukaemic infiltration does not take place; Rosenthal (1956) has expressed a similar view. If this view is correct, patients with disseminated tuberculosis and a leukaemoid reaction should develop frank leukaemia after effective anti-tuberculous treatment; such cases would establish the true nature of the leukaemoid reaction, but none has yet been described.

The absence of the usual cellular reaction at the rim of the tuberculous foci is common in cases of disseminated tuberculosis with associated blood changes. Arends (1950) claims that the absence of the usual cellular response and the associated blood changes might represent an inherent bone marrow defect. O'Brien (1954) postulates that both findings might be the result of an abnormal sensitivity of the body to the products of the tubercle bacillus.

Many cases of non-reactive tuberculosis were reported before the introduction of steroid therapy which is known to affect the local cellular response to infection. We find it impossible to decide whether the non-reactive lesions in the two cases reported here were the result of steroid therapy or would have developed had it been withheld.

Summary

Two cases, thought to have myeloid leukaemia, but found at post-mortem examination to have disseminated tuberculosis, are reported.

The diagnosis of disseminated tuberculosis is discussed.

The importance and difficulty of distinguishing between leukaemia and a leukaemoid reaction are stressed.

We wish to thank Privates J. Taylor and R. Spelman for technical and clerical assistance and Mr. D. E. Tomkinson for the photographs.

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OBSERVATIONS ON THE CENTRIFUGAL SEGREGATION OF YOUNG ERYTHROCYTES

A POSSIBLE METHOD OF GENOTYPING THE TRANSFUSED PATIENT

BY

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On certain occasions the need arises to discover the blood group genotype (more correctly phenotype) of a patient who has already been transfused. Some patients, for example, appear to form antibodies more actively than the general population, and in them it may be desirable to avoid transfusing all blood but that of the identical genotype. If they have already been transfused there is a mixture of cells in their circulation, and, even with a knowledge of donor genotypes, it may be impossible to uncover the patient's own contribution.

A more modern aspect of this problem is the attempt to treat hypoplastic anaemia or leukaemia with transfused haemopoietic cells. To give the maximum chance of success such cells should be of identical genotype except in so far as a single minor difference may be useful for the detection of foreign circulating cells, and is thereby an index of success.

In these circumstances, rather than wait three months or more until all previously transfused erythrocytes are eliminated, the following method may be tried. It depends, in essence, upon the centrifugal segregation of the younger and lighter circulating cells which, with certain exceptions later described, are derived from the subjects' own centres of haemopoiesis.

Evidence to show the possibilities and limitations of the method is presented, the data being of three kinds: (1) Serological analyses of known donor-recipient blood-group mixtures; (2) studies to show the concentration of reticulocytes by centrifugation; (3) studies on the gravitational behaviour of ^{51}Cr-tagged erythrocytes after transfusion.

Methods

For all studies in which ^{51}Cr-tagged cells were used 24 ml. of blood was collected into heparin. For serology and in some of the reticulocyte analyses 3.8% sodium citrate (0.5 ml. in 5 ml. blood) was the anticoagulant.

As soon as possible after collection 5–20 ml. of whole well-mixed blood was centrifuged for 20 minutes at 2,400 to 2,700 r.p.m. (c. 2,000 *g*). The plasma was removed and the upper quarter of the volume of packed red cells was carefully pipetted off. This constitutes the "first top layer." A pipette plunged to the bottom of the tube then removed the lower quarter of cells, the "bottom layer." An aliquot of the "first top layer" was resuspended in the plasma and spun as before but in a narrow tube. A "second top layer" is thus obtained, and from this, using a precipitin or haematocrit tube, a "third top layer" can be similarly derived. The quantity of this is enough for reticulocyte counts or agglutination tests but not for chromium counting.

Reticulocyte counts were performed on smears counterstained with May-Grünwald Giemsa. The number of cells counted ranged from 500 to 5,000 according to the abundance or scarcity of reticulocytes.

In the chromium experiments 20 ml. of blood in acid-citrate-dextrose was tagged with 60–100 μc. of ^{51}Cr. For valid comparison of the radioactivity of each sample the haemoglobin concentration was determined and the results (corrected for decay) are expressed first as counts/sec./g. Hb, and then this figure is converted to a percentage of the whole-blood activity of the first sample.

In the serological studies cells from whole blood and from each layer were washed in saline before being tested by standard agglutination techniques.

Absolute values were determined on fresh blood samples with sequestrene as anticoagulant.

Results

Serological Analyses of Known Donor-Recipient Blood Group Mixtures.—Eighteen patients were found who in the course of treatment received blood which had one or more detectable antigens lacked by the recipient. Following transfusion one to three samples of blood were taken at intervals and were tested with sera known to give negative results with the pure recipient blood.

TABLE I

ANALYSES OF BLOOD-GROUP MIXTURES AND EFFECT OF CENTRIFUGATION ON SUCH MIXTURES AT INTERVALS AFTER TRANSFUSION

Case No.	Recipient Group	Donor Group	Quantity (Units)	Storage	1	2	3
1	dd	D+	2	7 days	++, ++, ++, +, 2d	+++, +++, ++, ++ (1·2% retics), 5d	
2	♂♂, MM	E+(2) N+(2)	3	10–22 ,,	(E) +++, ++, W (N) ++, ++, W (42% retics) 3d		
3	dd	D+	6	7 ,,	+++, W, neg., neg., 1d	+++, W, neg., neg., 7d	
4	♂♂	E+	1	20 ,,	+, +, W, neg., 2d	+, W, W, neg. (10·2% retics), 10d	
5	♀♀	C+	4	14–18 ,,	++, ++, ++, 3d	+, +, +, ++ (8·9% retics), 7d	+, W, W, W (25% retics), 14d
6	dd	D+	2	5 ,,	++, W, W, neg. (10·3% retics), 3d	+, W, neg., neg., 10d	
7	♀♀	C+	1	?	++, W, neg., neg., 3d	++, W, W, neg., 9d	
8	dd	D+	1	15 ,,	++, ++, W, ++, 4d	+, +, +, +, 10d	+, +, +, +, 17d
9	dd	D+	2	8 ,,	+++, +, W, 3d	++, +, W, neg. (36·6% retics), 9d	
10	♀♀	C+	1	13 ,,	+++, +, +, +, 2d	++, +, +, W, 13d	++, +, W, neg., 21d
11	NN	MN	1	23 ,,	++, +, W, W, 6d	++, +, W, neg., 13d	
12	♂♂	E+	1	17 ,,	+, W, neg., neg., 6d	+, W, neg., neg., 14d	
13	MM	MN	1	?	++, +, W, W, 3d	++, W, neg., neg., 10d	++, W, W, W, 17d
14	NN	MM	1	20 ,,	+++, ++, ++, +, 3d	++, ++, ++, ++, 9d	++, ++, ++, +, 16d
15	MM	MN	2	11–14 ,,	++, +, W, W, 2d	++, +, +, W, 10d	
16	dd	D+	3	20 ,,	++, W, neg., neg., 1d	++, W, neg., neg., 4d	
17	MM	MN	2	14 ,,	+++, +, W, neg., 10d	+++, ++, +, W, 17d	
18	dd	D+	2	14–20 ,,	++, W, neg., neg. 2d	+++, W, neg., neg.,	++, +, neg., neg., 21
			3 (2d later)	10–16 ,,	+++, W, neg., neg. 7 (9d)	14d (16d)	(23d)

In each column of analysis is shown (left to right) the reactions in whole blood, "first top layer," "second top layer," "third top layer" (in some), and the interval in days after transfusion. The reticulocyte percentage refers to the most refined top layer.

The results are listed in Table I. The interval after transfusion is indicated for each test, and beside this the degree of agglutination is shown for the whole blood and successive "top layers." The figure for reticulocytes in six cases refers to the percentage of these cells in the most refined top layer tested. Three points deserve attention: (1) The length of storage time of the transfused blood is not a factor influencing the rate or degree of segregation. (2) The amount of blood given does not always determine the degree of agglutination in the mixture. Thus in Case 10 one bottle of C+ blood gave rise to a +++ agglutination two days later, whereas in Case 5 4 pints given with the same donor-recipient combination and tested with the same serum gave no more than a ++ reaction at three days fading to a weak agglutination at 14 days. In partial explanation it should be stated that the degree of agglutination was measured by the size of the agglutinates rather than by the proportion of free and agglutinated cells. The dosage effect of the C antigen may also be a relevant factor. (3) In 14 cases there was satisfactory separation of cells so that in the topmost layer the patient's true genotype was revealed. In four cases separation was inadequate although in two of these the "top layer" reaction for the foreign antigen was weaker than in the mixture. The lapse of time (two to five days in Case 1; three to 16 days in Case 14) did not appear to increase the chances of effective separation, and in Case 1 this may possibly be due to slow erythrocyte production during this period, the reticulocytes on the fifth day being 0.1% in whole blood, 1.2% in the "third top layer." In one of the other unresolvable cases (Case 8) time appeared to be a contrarily acting factor, initial separability giving way to a weakly reacting homogenous cell mixture. In Case 5 a uniform but weakening

TABLE II

CONCENTRATION OF RETICULOCYTES BY CENTRIFUGATION

Anti-coagulant	Reticulocyte Ratios: 1st Top Layer / Whole Blood		2nd Top Layer / Whole Blood		3rd Top Layer / Whole Blood		No. of Estimations	No. of Patients
	Range	Mean	Range	Mean	Range	Mean		
Citrate ..	1·0–3·5	2·4	2·8–8·0	5·1	7·0–16·0	11·1	38	21
Heparin ..	1·8–4·2	2·6	2·3–13·0	5·5	—	—	43	11

distribution of antigen was found despite a reasonable concentration of reticulocytes in the upper layers.

Separation of Reticulocytes.—Progressive concentration of reticulocytes can be achieved by the method of centrifugation described above. In 81 blood samples from 32 patients in which two or more top layers were analysed only one failed to show a conspicuous increase in the reticulocyte content. This example and eight typical results are illustrated in Fig. 1. Table II shows that there is some variation in the degree of concentration, but with the technique used the increase from stage to stage is roughly logarithmic. The variation occurs even in different samples from the same patient, but it appears to be irrespective of the initial number of reticulocytes. There was no obvious distinction in behaviour between cells with different amounts of cresyl-blue staining material.

Studies with ^{51}Chromium-labelled Erythrocytes.—Before describing the results it is necessary to explain the precise objective of these studies. An ordinary fresh blood sample labelled with ^{51}Cr will contain cells of all ages. After reinjection, as these cells age, any young cells in the blood will be unlabelled and there may

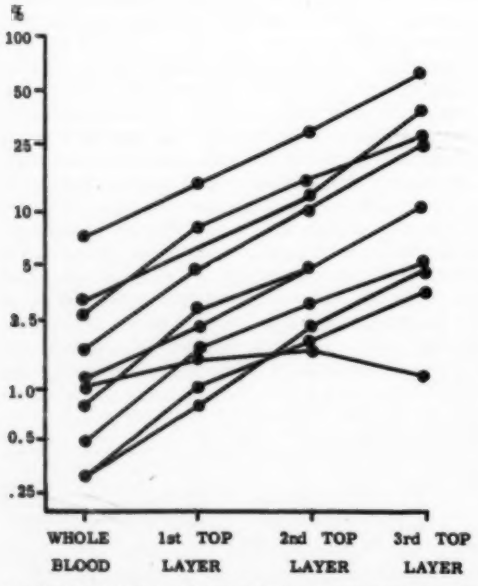


FIG. 1.—Concentration of reticulocytes by progressive centrifugation in nine typical cases and one exceptional case.

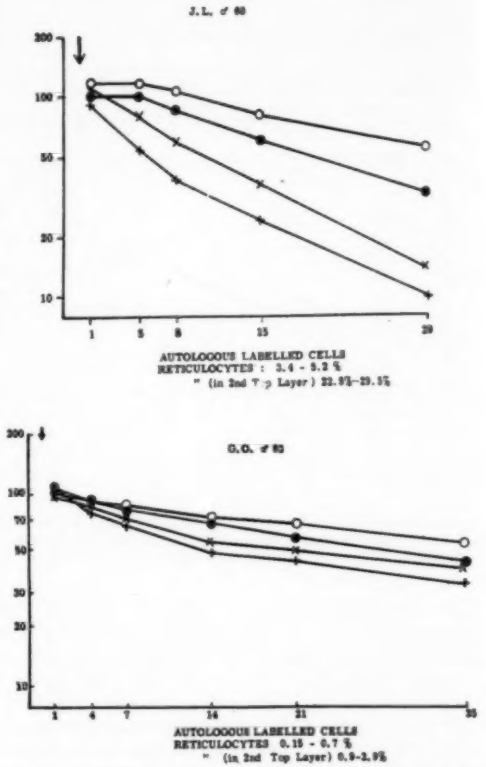


FIG. 2a, b.—Survival of ^{51}Cr-tagged autologous erythrocytes in two patients and the effect of centrifugation on the concentration of tagged cells. (Results expressed as a percentage of the initial whole blood activity in counts per second per g. Hb.) Whole blood, ●——●; "bottom layer", O——O; "first top layer," X——X; "second top layer," +——+.

thus appear on centrifugation an upper layer of cells free from radioactivity.

The cells chosen for labelling could be either autologous or from a donor, but, since our aim was to learn if the young cells from within a mixed population could be distinguished, all but two of these studies were made with donor blood, fresh or stored, being in six cases part of a larger transfusion. This, as will be seen, raises complications. Multiple transfusion, variations in storage time, and the fact that all 11 patients were suffering from malignant disease, haemorrhage, or surgical trauma must be taken into account.

The results of the experiments with autologous labelled cells are presented in Fig. 2. Both were

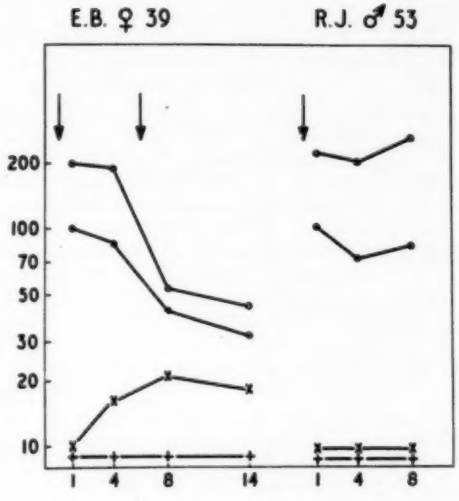


FIG. 3a and b

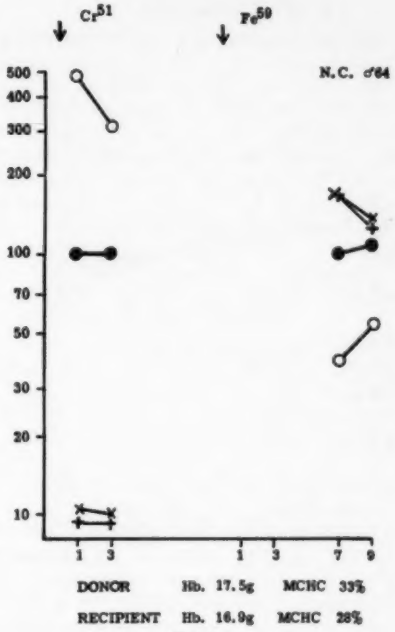


FIG. 3c

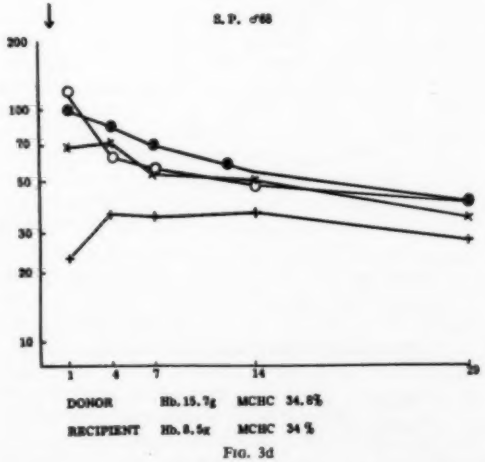


FIG. 3d

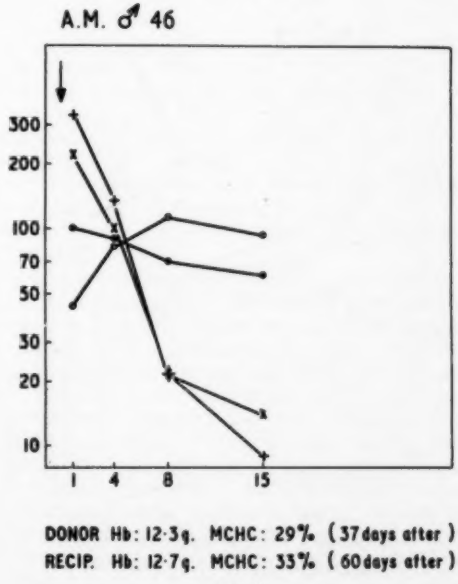
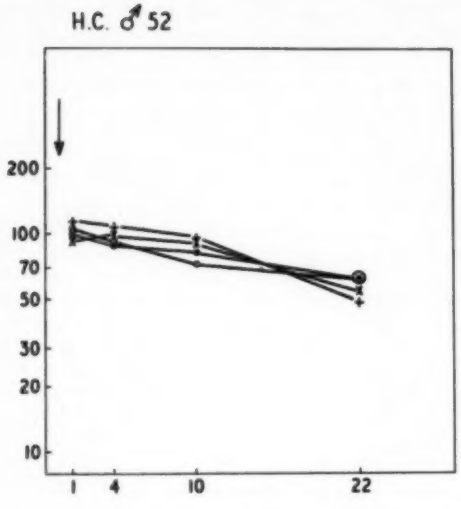


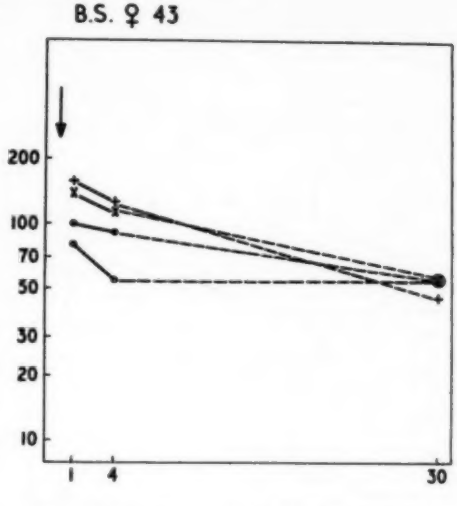
FIG. 3e

FIG. 3a–i.—Survival of ^{51}Cr-tagged donor erythrocytes in nine transfused patients.



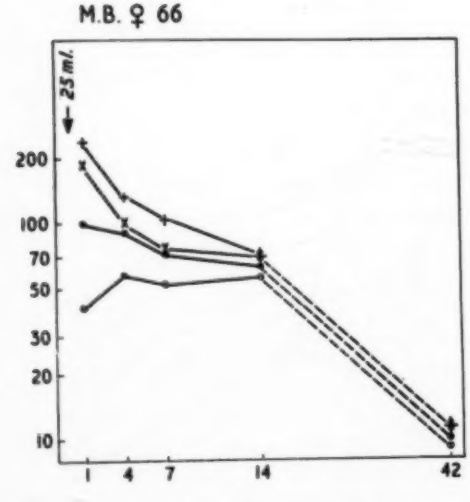
DONOR Hb: 15·1 g. MCHC: 33·5 % (60 days after)
RECIP. Hb: 17·3 g. MCHC: 33 % (90 days after)

FIG. 3f



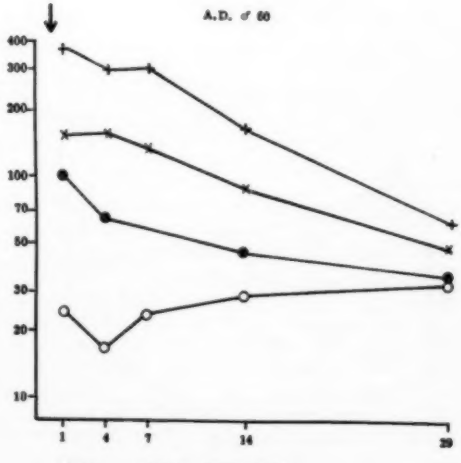
DONOR Hb: 12·4 g. MCHC: 32 % (80 days after)
RECIP. Hb: 12·2 g. MCHC: 33·5 % (70 days after)

FIG. 3g



DONOR Hb: 8·9 g. MCHC: 28 %
RECIPIENT Hb: 13·1 g. MCHC: 32 %

FIG. 3h



DONOR Hb 9.7g MCHC 29.5%
RECIPIENT Hb 14.9g MCHC 34%

FIG. 3i

cases of fairly advanced malignant disease. They were selected because, while similar in most respects, one (Fig. 2a) had consistently about 10 times as many reticulocytes as the other (Fig. 2b), a difference which is only explained in part by a shorter mean cell life, i.e., 50% elimination at 20 days compared with 28 days.

It is apparent that the higher reticulocyte concentration is associated with a more rapid clearing of labelled cells from the top layers, but changes must also be occurring in the immediate post-reticulocyte phase. Thus in Fig. 2a the ratio top layer activity/whole blood activity is constantly decreasing at least up to 15 days while a similar but lesser change is seen in Fig. 2b. Clearly the reticulocytes alone cannot be responsible for this alteration, and it suggests that the gain in density of the cells proceeds at an ever-decreasing rate which after about 15 days is very slow.

In the studies where donor cells were labelled (Fig. 3a–i) there can be distinguished three main groups of cases. In the first three cases (Fig. 3a–c) there is complete segregation of donor cells to the lower layers from the first (24-hr.) sample onwards, while at the other extreme are four cases (Fig. 3f–i) with no satisfactory clearing of the upper layers during the course of the experiments. In between come two cases, in one of which (Fig. 3d) the labelled cells are most concentrated in the middle layers while in the other (Fig. 3e) there is a rapid shift within two weeks from the upper to the lower layers.

Obviously some other hypothesis is needed to account for such very different results, and this may be partially provided by a consideration of the relative haemoglobin concentrations in the donor and recipient corpuscles. The values were determined for 17 out of 18 bloods, but in seven cases after the experiments. Clearly the latter data are of limited value, but, as there is no reason to suspect any great change since the time of the experiments, they are of some use if they confirm, as they do, the better-established data.

The evidence, so far as it goes, shows that the initial segregation is in accordance with the relative M.C.H.C. of donor and recipient bloods. On the first day, that is, the cells with the higher M.C.H.C., and hence presumably heavier, whether of donor or recipient origin, are more concentrated in the bottom layers. Thereafter, in some cases (especially, for example, Fig. 3e), an adjustment of some sort may occur to change this relationship in addition to the ageing effect previously noted.

Discussion

It might be thought a tautology to say that the centrifugal behaviour of a cell depends upon its density, but, in fact, this is not always true. If sedimentation is slow, as under the influence of gravity only, there is time for aggregation of a proportion of cells to occur, and this event, which depends upon surface characteristics, will cause the more rapid precipitation of aggregated cells. Simple sedimentation was, indeed, found by Stephens (1939) to be a useful way of separating young, less readily aggregated erythrocytes, but in the different circumstances of our experiments this method has proved ineffective.

Most workers (for references see Marks and Johnson, 1958), relying on density differences, have used centrifugation with or without the addition of albumin to the suspending medium. The factors which render this separation effective are:

(1) The greater water content of the young cell which is reflected in its greater size with consequent dispersal of protein and other material.

(2) The absolute increase of haemoglobin in the mature cell, which one must assume, since haemoglobinization is still occurring in the reticulocyte (Walsh, Thomas, Chow, Fluharty, and Finch, 1949).

(3) A decrease in lipid content of the maturer cells (Prankerd, 1958).

Borun, Figueroa, and Perry (1957) using ^{59}Fe have produced some interesting data indicating that density is, in fact, a function of erythrocyte age but that considerable individual variation occurs in the degree of this relationship and also in its timing. Nevertheless it is clear that the greatest increase in density occurs early in the life of the cell.

Bearing in mind these sources of variation, it is scarcely surprising that one cannot, when dealing with cell mixtures, always rely on centrifugation to bring the young erythrocytes to the top. But our studies show that the most adverse conditions for separation are those where cells with a low haemoglobin concentration are transfused to a recipient of more fully haemoglobinized cells. Luckily this is the opposite of what usually occurs in clinical practice (our experiments with chromium-labelled cells are heavily biased by selection), and, as shown in the serological studies, in the majority of cases the recipient genotype is revealed in the top layer.

The method has been successfully used in one patient of unknown genotype, and the test proved

correct after a lapse of time when all transfused cells had been eliminated; also in one case to detect erythrocyte chimerism as a result of transfusion of haemopoietic cells. By testing the young cells as they emerge into the circulation the development and cessation of function of donor marrow cells can be followed with much greater precision than could ever be obtained by the use of whole blood alone.

Alternative procedures should be mentioned. Cathie (1946) has described the genotyping of bone marrow cells, and another approach to the problem is suggested by the work of Marks and Johnson (1958) whereby the osmotic resistance of young erythrocytes could be exploited. Both methods would seem to have their own difficulties. Meanwhile those who find occasion to employ centrifugal separation should take note of the pitfalls.

Summary

Observations have been made on the separation of erythrocytes by centrifugation. Young cells being lighter tend to spin to the top, but in mixtures caused by transfusion other factors, especially the corpuscular haemoglobin concentration, may sometimes obscure this tendency. Nevertheless centrifugation can be recommended at least as an adjunct in the problem of genotyping cell mixtures.

We are indebted to Mrs. E. M. Ledlie for the chromium determinations, to Dr. A. E. Mourant and Dr. R. A. Zeitlin for typing sera, and to Dr. D. M. Farkin for helpful criticism.

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J. clin. Path. (1959), **12**, 319.

PURE RED CELL ANAEMIA COINCIDENT WITH BENIGN THYMOMA

BY

F. KURREIN

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(RECEIVED FOR PUBLICATION NOVEMBER 12, 1958)

Pure red cell agenesis in adults was first described by Kaznelson (1922). Since then the condition has been seen with and without coincident thymic tumour, and by March, 1958, 14 cases of the tumour-anaemia syndrome had been reported (Davidsohn, 1941; Humphreys and Southworth, 1945; Chediak, Fuste, and Rosales, 1953; Ross, Finch, Street, and Strieder, 1954; Chalmers and Boheimer, 1954; Bakker, 1954; Stibbe, 1955; Weinbaum and Thompson, 1955; Ramos and Loeb, 1956; Bayrd and Bernatz, 1957; Mielke, 1957).

In view of the comparative rarity and the numerous problems posed by this condition publication of another single case seems justified.

Mrs. L. W., aged 70, was first seen in September, 1954, when she was referred to the chest clinic for investigation of persistent purulent bronchitis. Chest radiographs showed a midline retrosternal mass about 7 cm. in diameter. Tomography and bronchoscopy before and after the induction of a pneumothorax confirmed the extrapulmonary site of the mass. As the possibility of malignancy could not be ruled out surgery was offered, but the patient refused operation. A blood count at this time showed a haemoglobin of 11.7 g.%, and 16,000 white cells per c.mm. The stained film presented a normal picture.

The patient was kept under observation, attending at three-monthly intervals. Eight sets of radiographs taken over two years failed to show any change in the retrosternal mass. Mrs. W.'s clinical state varied only with the relapses in the bronchitis, which were controlled by courses of terramycin. On her last out-patient attendance (September 24, 1956) she had a little white sputum, but looked and felt well.

On November 22 she was admitted with a history of severe dyspnoea, palpitations, and swelling of the legs coming on in the last few weeks. Furthermore, her urine had become thick and foul smelling.

Examination showed her to be grossly anaemic, slightly cyanosed, and dyspnoeic on movement. Apart from a few rales in the chest and the swelling of the legs there were no abnormal physical signs (blood pressure 150/70 mm. Hg).

Investigation centred on the anaemia. Haemoglobin was 4.5 g.% with normal-looking cells in the stained film. White cells numbered 14,500 per c.mm. The correct diagnosis was suggested by the bone marrow films, which showed only one erythroblast, and that dubious, in a count of 1,000 nucleated cells. Megakaryocytes and the entire white cell series looked normal. In addition there were fair numbers of small, dense, "lymphoid" cells (Fig. 1) similar to those described in previous cases (Chalmers and Boheimer, 1954; Bayrd and Bernatz, 1957). Repeated reticulocyte counts failed to produce any evidence of red cell regeneration. Serum bilirubin was 1 mg.%, the direct and indirect Coombs tests were negative. No agglutinins could be found in normal and acidified serum. Gastric content analysis showed a low free acid curve. No free or occult blood was

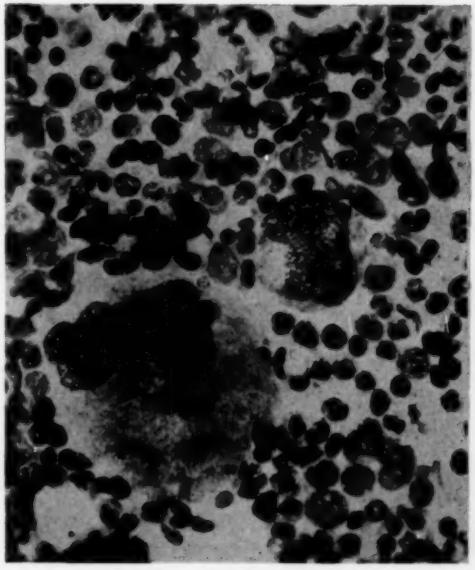

FIG. 1

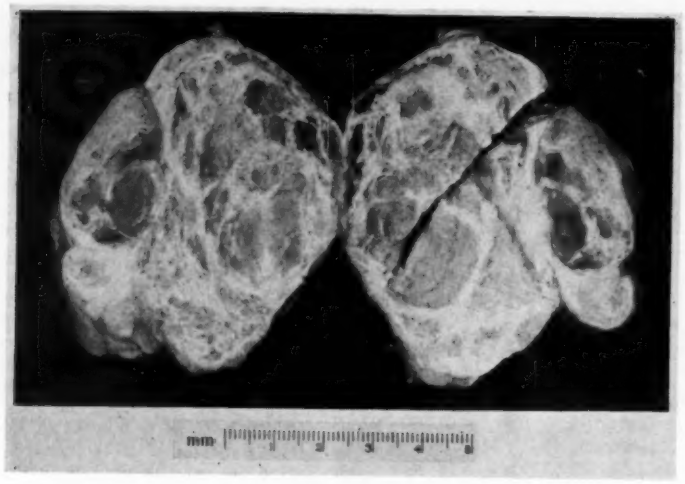

FIG. 2

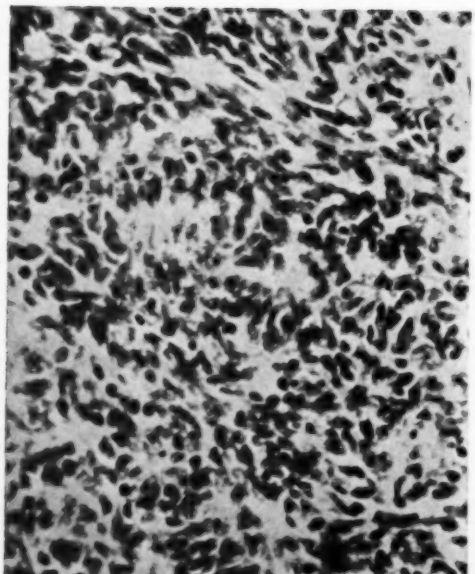

FIG. 3 × 300.

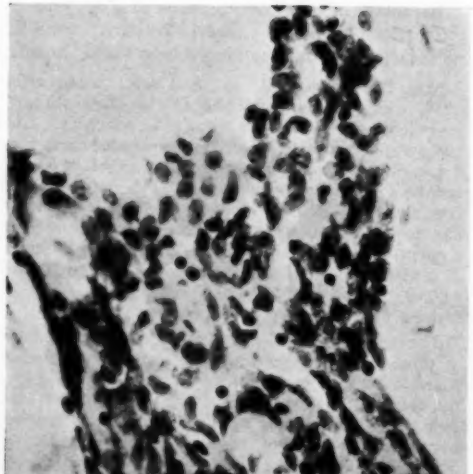

FIG. 4 × 300.

demonstrated in faeces or gastric juice. Repeated urine cultures confirmed infection by *B. proteus* and a coliform bacillus.

The results of blood counts during the last two years are tabulated below:

Date	Hb (g.%)	W.B.C. per c.mm.	E.S.R. (Westergren)	Morphology in Stained Film
13/9/54	11·7	16,000	40	Normal
26/11/54	14·5	7,000	5	,,
18/4/55	14·8	12,000	51	,,
21/11/56	4·5	14,500		,,
3/1/57	3·5	31,000		,,

In the four weeks after admission the anaemia was treated with 4 pints of whole blood and 2,450 mg. of cortisone. Transfusions were kept to a minimum because of respiratory embarrassment due to the exacerbated bronchitis. A repeat bone marrow examination at this time showed unchanged appearances. The respiratory and urinary disease processes proceeded and the patient died on January 4, 1957.

Post-mortem examination on January 7 yielded the following positive findings.

There were old, adherent thrombi in the pulmonary arteries, the left renal vein, and in the pelvic venous plexus, but none in the limb veins. The superior mediastinum contained an encapsulated tumour, weighing 48.5 g. and measuring 7×6×2.5 cm., which shelled out easily from the loose connective tissue and pleural margins (Fig. 2). Externally it was somewhat bossed, while the cut surfaces showed a tan-coloured, firm tissue divided by fibrous septa and areas of calcification. The lungs were patchily adherent to the chest wall and showed tubular bronchiectasis and emphysema. The bone marrow of the sternum, ribs, lumbar vertebrae, and femur was soft, abundant, and greyish-brown. The genito-urinary tract presented some simple, uterine fibroids and a severe, chronic cystitis.

The liver was normal in size and shape, weighing 1,200 g. The spleen looked normal and weighed 150 g. Lymph nodes were unremarkable except in the hila of the lung, where they showed oedema and reactive hyperplasia. The adrenals, pituitary, and thyroid looked normal.

Microscopy of the tumour revealed islands of plump oval or spindle cells with large vesicular nuclei. These cells were arranged in irregularly sized and shaped areas separated by densely hyalinized or calcified collagenous septa. Loose aggregates of small round cells consistent with lymphocytes occurred among the large cell islands. There were also a number of cyst-like structures apparently derived from the oval cells. No bodies resembling Hassall's corpuscles were seen (Figs. 3 and 4). The appearances were those of a benign lympho-epithelioma of the thymus.

Sections of liver and spleen showed a fair amount of iron giving Perls's reaction. No such iron was found in the bone marrow. The tumour did not contain any stainable iron.

Comment

This case resembles most of the previously reported ones in many or all the relevant features. There was unequivocal evidence of a stationary mediastinal tumour long before the abrupt onset of the anaemia. Here, too, was failure to respond to medical treatment (surgery was contraindicated by the general condition and the age of the patient). No haemolytic or immune mechanism could be demonstrated.

In this instance the process was confined to the red cells without, even at the end, involvement of platelets or leucocytes. Although the patient was old, neither clinically nor at necropsy was there anything to suggest failure or overactivity of any of the known hormone-producing organs. If the thymic tumour was indeed the source of a hormonal influence on the marrow no explanation can be given for its action at the time it occurred, although the tumour was known to be present for at least two years previously.

The cases of Opsahl (1939) and Radojević and Hahn (1935) have been excluded from this review because of the doubtful nature of the thymic tumours, and Matras and Priesel's case (1928) was not a truly proven agenesis.

Summary

A further fatal case of red cell agenesis coincident with a benign thymoma is reported.

I am indebted to Drs. C. F. Cropper and E. N. Moyes for access to their clinical records. This paper would not have been written without the help and encouragement of Drs. J. N. Marshall Chalmers and P. Kidd. The photomicrographs are by Mr. G. D. Holland.

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THE SIGNIFICANCE OF BLUE POLYCHROMASIA

BY

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(RECEIVED FOR PUBLICATION JULY 12, 1958)

The standard references and textbooks comment on the spectrum of colours, from grey to blue, seen in the polychromasic erythrocytes. The significance attached to these cells varies, and the general attitude is summarized by Wintrobe (1956) in the following statement:

"Macrocytes, and to a lesser extent polychromatophilia, are like reticulocytes, manifestations of orderly and probably efficient new blood formation."

The present study suggests that the significance of polychromatophilia is in part determined by the colour of the polychromasic cell.

Materials and Methods

As the polychromasic cells (Key, 1921; Rodan, 1949) and nucleated red cells (Stein, 1948; Rodan, 1949) collect in the buffy layer, a blind study was carried out on the buffy layer from 95 patients, including 11 children under the age of 6 years. A total of 137 slides (no more than two from each patient) was examined. With one exception (an iron deficiency anaemia) all slides examined represented the morphology of the red cells before treatment.

The buffy layer was lifted with a Pasteur pipette from the standard haematocrit tubes. The contents of the pipette was divided between two slides and the excess plasma and red cells drained off with a piece of blotting paper placed at the edge of the drop. In draining the drop to almost complete dryness, the small grey-white granules of buffy material were left adherent to the glass slide. Smears made from these granules were stained with May-Grünwald Giemsa stain (Dacie, 1956).

The characteristics—blue polychromasia, grey polychromasia, and nucleated red cells—were estimated independently using the following scale:

Trace (Tr)=occasional cells only.
+ =occasional cells in every oil-immersion field.
++ =few cells in each field.
+++ =cells in 25% of each field.

Reticulocyte counts were carried out using 1% brilliant cresyl blue in saline (Dacie, 1956). A total of 2,000 cells was counted in each slide.

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Results

The quantitative assessment of grey and blue polychromasia is presented in Table I.

TABLE I
ASSESSMENT OF GREY AND BLUE POLYCHROMASIA

Number of Slides	Megaloblastic Anaemia	Haemolytic Anaemia	Myelophthisic Anaemia	Fe Deficiency Anaemia	Thrombocytopenia	Normal	Miscellaneous
Polychromasia "Grey"							
0	6	3	8	2	9	9	9
Tr							
+	14	11	10	10	1	4	3
++	8	4	3	0	2	0	0
+++	4	4	2	0	1	0	2
Polychromasia "Blue"							
0	9	18	16	11	10	13	11
Tr	4	0	1	1	1	0	1
+	11	2	2	0	2	0	0
++	4	1	3	0	0	0	1
+++	4	1	1	0	0	0	1

Bold figures = children.

Polychromasia Grey.—One plus polychromasia was found in the buffy layer of normal individuals. More than one plus was found in all other groups with the exception of the iron-deficiency anaemias. Patients exceeding one plus in the thrombocytopenic and miscellaneous group were all children. Thus in the adult more than one plus grey polychromasia was found in the megaloblastic, myelophthisic, and haemolytic anaemias.

Polychromasia Blue. — Blue polychromasia was not found in the buffy layer of normal individuals. In the iron-deficiency anaemias a single blue polychromasic cell was found in the buffy layer of a patient receiving iron therapy. In the thrombocytopenic and haemolytic groups these cells were found in children only. Thus in the adult more than a trace of blue polychromasia was found in the megaloblastic, myelophthisic anaemias as well as in a patient suffering from an aplastic anaemia (miscellaneous group).

There appears to be a direct relationship between the reticulocyte count and grey

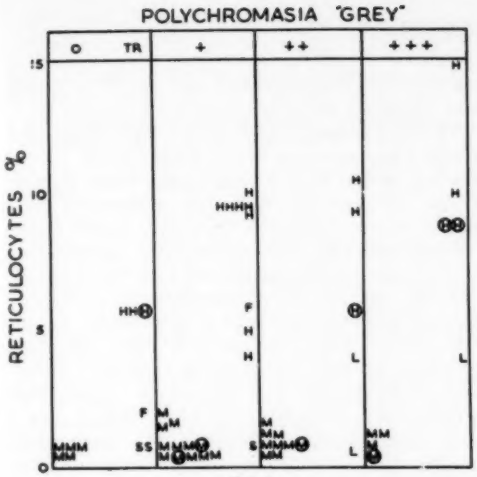

FIG. 1

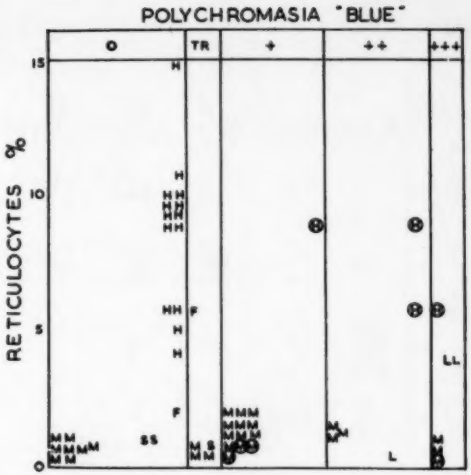

FIG. 2

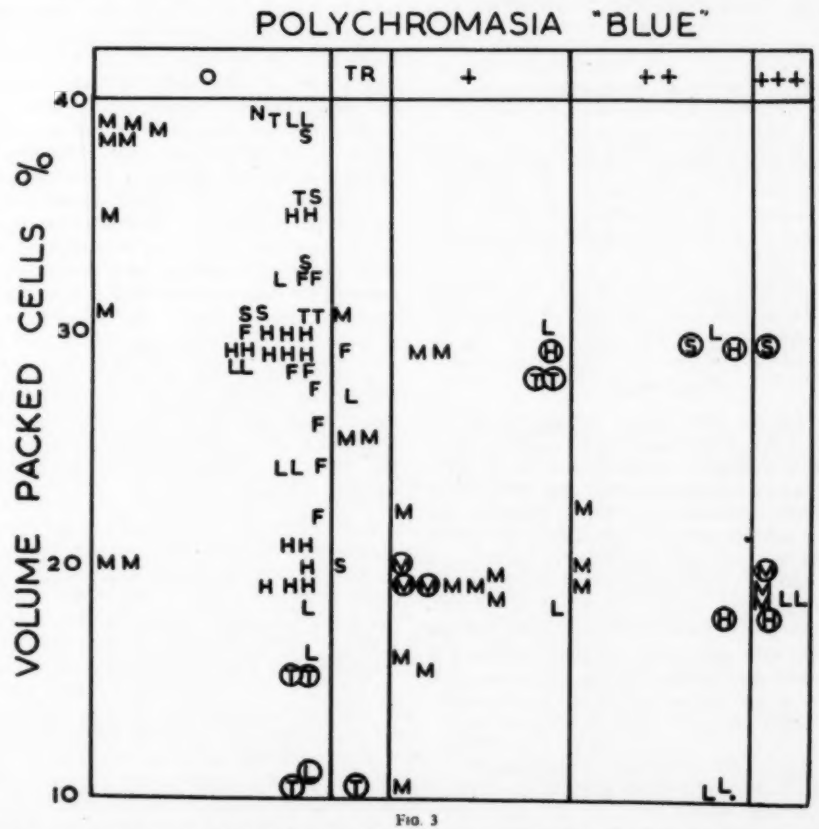

FIG. 3

H—Haemolytic anaemia. M—Megaloblastic anaemia. F—Iron-deficiency anaemia. T—Idiopathic thrombocytopenic purpura. L—Myelophthisic anaemia. S—Miscellaneous group. Encircled letters—children. N—Normal.

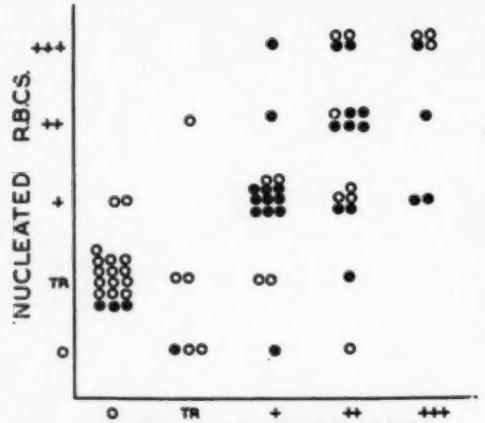

FIG. 4

●—megaloblastic anaemia. O—all other groups.

polychromasia in the haemolytic anaemias (Fig. 1).

In the adult there appears to be an inverse relationship between blue polychromasia and the reticulocyte count (Fig. 2).

In the megaloblastic group the incidence of blue polychromasia is related to the severity of the anaemia (Fig. 3).

There appears to be a direct relationship between the incidence of blue polychromasic cells and the nucleated red cells (Fig. 4).

Discussion

The basophilic material in the polychromasic cell is a ribonucleoprotein (Haenel, 1949) which on contact with brilliant cresyl blue is precipitated into the filamentous flakes seen in the reticulocyte (Orten, 1934). The present study suggests that when blue polychromasia is present in the adult, then a low reticulocyte count can be anticipated and a serious disturbance of haemopoiesis suspected in the form of a megaloblastic, myelophthisic, or aplastic anaemia. Although the division between grey and blue polychromasia is vague repeated observations on the same slide indicate that blue polychromasia can be identified and estimated fairly accurately.

The relationship between polychromasia and reticulocytosis can be briefly summarized. In the normal person with a reticulocyte count ranging from 0.2 to 2% only an occasional grey polychromasic cell is seen in the peripheral blood. With the response to haemolysis and haemorrhage there is an increase in the reticulocyte count (R) and the number of polychromasic cells (P). In the adult the latter cells are confined to the grey type, whereas in the child and infant large numbers of the blue type may also be found. This orderly regenerative response is associated with a slight increase in the P/R ratio.

When erythropoiesis is profoundly disturbed, as in the megaloblastic, myelophthisic and aplastic anaemias, there is an increase in the number of polychromasic cells without a corresponding rise in the reticulocyte count and the P/R ratio rises markedly. In the adult this is associated with the appearance of blue polychromasic cells in the peripheral blood.

Dacie (1954) comments on the fact that large numbers of normoblasts in the peripheral blood (except in young children) "should cause the observer to reconsider the diagnosis of primary haemolytic anaemia." The correlation between blue polychromasia and the nucleated red cells suggests that a similar significance should be attached to the presence of blue polychromasia.

Summary

It is suggested that there is a difference in the significance of grey and blue polychromasia in the adult peripheral blood and that the latter cell suggests a serious disturbance of haemopoiesis in the form of a megaloblastic, myelophthisic, or aplastic anaemia.

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THE COAGULATION PROPERTIES OF HUMAN ERYTHROCYTES

BY

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A thromboplastic factor has been demonstrated in human red cells by Shinowara (1951), Ottaviani, Dettori, and Manai (1954), Quick, Georgatsos, and Hussey (1954), Leupold (1955), Bounameaux (1957a), and Georgatsos, Hussey, and Quick (1955), and named the "erythrocyte clotting factor" (E.C.F.). Its activity is similar though not identical with that of platelet factor 3. Thromboplastic activity is not produced by whole erythrocytes, but only after lysis by freezing or dilution with distilled water. Lysed erythrocytes increase the prothrombin consumption of platelet-poor or platelet-rich plasma and restore to normal the prolonged recalcification time and the impaired prothrombin consumption of a thrombocytopenic plasma (Georgatsos *et al.*, 1955). The E.C.F. activity depends on the presence of thromboplastinogen (Izarn, Hussey, and Quick, 1955). The increase of prothrombin consumption of a platelet-free haemophilic plasma, to which thromboplastinogen is added, is higher when platelets are replaced by lysed erythrocytes, when there is a linear relationship between this increase and the amount of thromboplastinogen.

The prothrombin consumption test in the presence of lysed erythrocytes, as suggested by Izarn *et al.* (1955), should therefore be very sensitive in detecting small quantities of thromboplastinogen or antihaemophilic globulin (A.H.G.) and it should be useful in differentiating between mild and severe A.H.G. deficiencies.

It was also shown by Leupold (1955), Georgatsos *et al.* (1955), and Bounameaux (1957a) that lysed red cells can be substituted for platelets in the thromboplastin generation test of Biggs and Douglas (1953).

This paper records an investigation into the coagulation properties of lysed erythrocytes. The possible interference of lysed erythrocytes in the various stages of the clotting process has been investigated and a study made as to whether other activities might be detected in red cells comparable with those already found in platelets by Deutsch, Johnson, and Seegers (1955). A preliminary report on the antiheparin activity of lysed erythrocytes has been published (Serafini and Centurelli, 1957).

Methods

Preparation of Lysed Erythrocytes (Haemolysate).—Nine volumes of human blood, taken into siliconed glassware after a clean venepuncture from a healthy person, were mixed with one volume of 0.1 M sodium oxalate and then centrifuged at 800 r.p.m. for five minutes. The platelet-containing plasma was pipetted off and the packed red cells were washed eight times with buffered saline, which, after slow centrifugation, was removed. After the last washing, red cells were centrifuged at 5,000 r.p.m. for 30 min. and the supernatant saline was removed. A concentrated haemolysate, obtained by freezing at −20° C. for 12 hours, was obtained from a suspension of 8–10,000,000 red cells per c.mm., containing only 500–2,000 platelets or their fragments per c.mm. After thawing, the haemolysate was stored at −20° C. and warmed to 37° C. before the experiments. Different dilutions of haemolysate in buffered saline (imidazole buffer at *p*H 7.3) were used.

Thromboplastic Activity.—This was assayed by the prothrombin consumption test of Merskey (1950) on 0.4 ml. of platelet-poor human oxalated plasma, to which 0.2 ml. of different concentrations of haemolysate or saline or of a platelet suspension and 0.2 ml. of 0.050 M $CaCl_2$ were added.

The thromboplastic activity of the haemolysate was also assayed by the thrombin generation test of Pitney and Dacie (1953), modified by O'Brien (1955), and by the thromboplastin generation test of Biggs and Douglas (1953).

The prothrombin consumption test of blood from cases of haemophilia or Christmas disease to which haemolysate has been added before clotting was tested by the method of Quick and Favre-Gilly (1949).

Antiheparin Activity. — Platelet-poor plasma samples containing different quantities of heparin (0.25 to 1 I.U. per ml.) were mixed with equal volumes of haemolysate or with a platelet suspension of 300,000 per c.mm. or buffered saline as control.

After incubation for 15 min. at room temperature, a constant volume of a thrombin solution ("thrombin," Roche) in imidazole buffer at *p*H 7.3 was added and the clotting time recorded. The present method does not depend on the erythrocyte thromboplastic activity and measures only the ability of the test material to neutralize the antithrombin activity of heparin.

Effect on Thrombin-fibrinogen Reaction.—Samples of a fibrinogen solution ("fibrinogen," Behring), containing 0.2 g. per 100 ml. in buffered saline at *p*H 7.3, were mixed with equal volumes of increasing concentrations of haemolysate or of a platelet suspension of 300,000 per c.mm. or saline as control. A constant volume of a thrombin solution in imidazole buffer was added and the clotting times recorded.

Factor V and Factor VII Activity.—Factor V was assayed by the method of Quick and Stefanini (1948) using oxalated human plasma stored for 18 hours at 37° C. as a substrate according to Wolf (1953), and factor VII by the method of Koller, Loeliger, and Duckert (1951). In both methods test plasma was replaced by haemolysate or controls.

Test for Factor VII Inhibition.—Factor VII activity of a normal serum was tested by the method of Koller *et al.* (1951) before and after incubation at 37° C. for 30 min. with increasing concentrations of haemolysate or controls.

Quick Times and Stypven Times.—The effect of different concentrations of haemolysate on the clotting time of a normal plasma, to which brain thromboplastin or Russell viper venom ("stypven," Burroughs Wellcome) diluted 1 in 3 in saline, and then 0.025 M $CaCl_2$ added, was investigated.

Tests for Anti-brain Thromboplastin and Anti-plasma Thromboplastin Activity.—The method of Tocantins (1943) for antithromboplastin activity was modified. Brain thromboplastin was incubated with haemolysate for 15 min. at 37° C. and then added to normal plasma, together with 0.025 M $CaCl_2$. Alternatively brain thromboplastin was incubated with haemolysate and normal plasma for 15 min. at 37° C. and then the mixture was recalcified. Clotting times before and after incubation were compared.

An inhibiting effect on plasma thromboplastin was investigated adding undiluted haemolysate, or buffered saline as a control, to the mixture of a normal thromboplastin generation test soon after the shortest clotting time of the substrates was reached. The rate of progressive decrease of plasma thromboplastin activity during incubation with haemolysate or with saline was investigated.

Test for Clot-retraction Promoting Activity.—Clot retraction of samples of platelet-poor plasma, to which different concentrations of haemolysate or controls (platelets or saline) and $CaCl_2$ were added in siliconed graduated tubes, was assayed by the method of Macfarlane (1939).

Results

Thromboplastic Activity of Haemolysate.—This is shown either in the thrombin generation or in the prothrombin consumption of a platelet-poor plasma to which haemolysate at different concentrations was added (Table I). A 0.2% concentration of haemolysate is still effective. A slight inhibitory effect of the less dilute haemolysate was observed in both tests, but chiefly on the prothrombin consumption.

Lysed red cells can be substituted for platelets in the test of Biggs and Douglas. The greatest activity is obtained by a 10% concentration of haemolysate; an excess decreases thromboplastin formation (Fig. 1). No thromboplastin is generated when haemolysate is substituted for either $BaSO_4$-adsorbed plasma or normal serum in the test. The activity of whole erythrocytes, if they are used in the thromboplastin generation test instead of haemolysate, is very poor, depending probably only on the few red cells damaged or lysed during washing (Table II).

The activity of haemolysate is stable and does not decrease after heating at 60° C. for 30 min. or after storage at 37° C. for a week or at 4° C.

TABLE I

THROMBOPLASTIC ACTIVITY OF HAEMOLYSATE AND INHIBITORY ACTION OF HIGHEST CONCENTRATIONS

Reagents	Thrombin Generation Test: Clotting Time (min.)	Thrombin Generation Test: Clotting Time (sec.)	Thrombin Generation Test: Time Elapsed (min.)	Thrombin Generation Test: Time Elapsed (sec.)	Fibrinogen Time (sec.)	Prothrombin Consumption (After 1 hr. at 37° C.) Serum Time (sec.)
Platelet-poor plasma, haemolysate (undiluted) + $CaCl_2$ ..	1	56	2	15	13·5	205
,, ,, ,, diluted 1 in 2 + $CaCl_2$ ..	1	48	2		13·5	—
,, ,, ,, ,, 1 ,, 5 ,, ..	1	35	2		11	287
,, ,, ,, ,, 1 ,, 10 ,, ..	1	30	2		11·5	419
,, ,, ,, ,, 1 ,, 25 ,, ..	1	38	2		12·5	—
,, ,, ,, ,, 1 ,, 50 ,, ..	1	55	2	30	15	154
,, ,, ,, ,, 1 ,, 100 ,, ..	2	35	4	15	17	46
,, ,, ,, ,, 1 ,, 500 ,, ..	2	55	4	30	24	27
,, ,, saline + $CaCl_2$	3	50	5	45	67·5	14
Platelet-rich plasma, ,, ,, ...	1	42	2	30	12	184

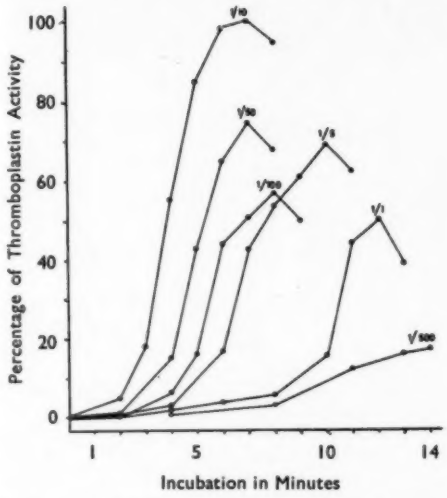

FIG. 1.—The thromboplastic effect of different dilutions of haemolysate in saline as substitutes for platelets in the thromboplastin generation test. Thromboplastin generation is inhibited with the highest concentrations of haemolysate.

for a month. It persists also after treatment with $BaSO_4$, but disappears after extraction by lipid solvents such as acetone and chloroform.

Cases of severe haemophilia or Christmas disease may be diagnosed by the thromboplastin generation test performed with haemolysate as a substitute for platelets. Normal results can be obtained in cases of partial A.H.G. deficiency. The prothrombin utilization of blood from mild

TABLE II

SOME PROPERTIES OF ERYTHROCYTE THROMBOPLASTIC ACTIVITY STUDIED BY THROMBOPLASTIN GENERATION TEST

Reagents	Results*	
	Maximal Thromboplastin Generation (sec.)	Incubation Time Elapsed (min.)
Normal $BaSO_4$-plasma, serum, and platelets + saline + 0·025 M $CaCl_2$ ..	12	6
Normal $BaSO_4$-plasma and serum + haemolysate (diluted 1 in 10) + saline + $CaCl_2$	11	6
Normal $BaSO_4$-plasma, serum, and platelets + haemolysate (diluted 1 in 10) + $CaCl_2$	10	6
Normal $BaSO_4$-plasma and platelets + haemolysate (diluted 1 in 10) + saline + $CaCl_2$	63	25
Normal serum and platelets + haemolysate (diluted 1 in 10) + saline + $CaCl_2$	54·5	26
Normal $BaSO_4$-plasma and serum + haemolysate (diluted 1 in 10) + 0·025 M $CaCl_2$	10	6
In the above mixture, haemolysate replaced by $BaSO_4$-treated haemolysate	10	6
In the same mixture, haemolysate replaced by whole erythrocytes ..	45	17
Normal $BaSO_4$-plasma and serum + haemolysate stored for 7 days at 37° C. + $CaCl_2$	9·5	5
In the above mixture, haemolysate stored for one month at 4° C. is used	10	5
In the above mixture, haemolysate is replaced by a suspension of powdered acetone-chloroform-extracted erythrocytes	48	12
Normal $BaSO_4$-plasma and serum + haemolysate (diluted 1 in 10) + $CaCl_2$	10·5	7
In the above mixture, haemolysate heated at 60° C. for 30 minutes and diluted 1 in 10 is used	12	10
In the same mixture, heated haemolysate (diluted 1 in 15) is used	11	7

* The shortest clotting time of substrates in each test is shown in the first column and in the second is the incubation time elapsed till the shortest clotting time was obtained.

TABLE III

USE OF HAEMOLYSATE IN TESTS FOR DETECTING SEVERE AND MILD ANTIHAEMOPHILIC GLOBULIN AND CHRISTMAS FACTOR DEFICIENCIES

Case No.	Diagnosis	Serum Prothrombin Activity (% Plasma Prothrombin) of 0·9 ml. Patient's Blood				Thromboplastin Generation Test					
						Using Platelet Suspension		Using Haemolysate Diluted			
								1 in 10		1 in 50	
		+0·1 ml. Saline	+0·1 ml. Haemolysate Diluted			Maximal Thromboplastin Generation (sec.)	Time Elapsed (min.)	Maximal Thromboplastin Generation (sec.)	Time Elapsed (min.)	Maximal Thromboplastin Generation (sec.)	Time Elapsed (min.)
			1 in 3	1 in 10	1 in 50						
1	Haemophilia	82	—	58·5	—	37 (N. 10)	17 (N. 5)	39 (N. 10)	19 (N. 5)	—	—
2	,, ...	72	—	50	—	—	—	41 (N. 12)	21 (N. 7)	—	—
3	Mild haemophilia .. (A.H.G. level = 5%)	58	9	14	32	—	—	—	—	—	—
4	Mild haemophilia .. (A.H.G. level = 12%)	36·5	18	21	27	28 (N. 12)	6 (N. 4)	22 (N. 11·5)	15 (N. 7)	—	—
5	Mild haemophilia .. (A.H.G. = 15%)	44	—	5·7	9·5	18 (N. 12)	6·5 (N. 5)	11·5 (N. 10)	5 (N. 5)	15·5 (N. 11·5)	5·5 (N. 5)
6	Christmas disease ..	55	25	30	—	37 (N. 12)	9 (N. 4)	19 (N. 11·5)	11 (N. 7)	—	—

N. = Normal values (in brackets).

cases of haemophilia to which haemolysate was added before clotting was restored to normal, but it was only improved, though remaining abnormal, in blood from patients with severe haemophilia or Christmas disease (Table III). These results agree with the observations of Georgatsos *et al.* (1955) and Izarn *et al.* (1955) that the thrombin generation of a haemophilic plasma is increased when haemolysate is substituted for platelets in the test of Pitney and Dacie (1953) (Table IV).

TABLE IV

USE OF HAEMOLYSATE AS A SUBSTITUTE FOR PLATELETS IN THROMBIN GENERATION TEST FOR DETECTING A.H.G. DEFICIENCY

Reagents	Clotting Time		Time Elapsed (min.)	Fibrinogen Time (sec.)
	min.	sec.		
Normal plasma + platelet suspension + 1/40 M $CaCl_2$	1	39	2	12
Normal plasma + saline + 1/40 M $CaCl_2$	4	7	10	51
Haemophilic plasma + platelet suspension + 1/40 M $CaCl_2$	6	25	13·5	39
Haemophilic plasma + saline + 1/40 M $CaCl_2$	9	40	17	98
Normal plasma + haemolysate (diluted 1 in 10) + 1/40 M $CaCl_2$	2	5	3·5	13·3
Haemophilic plasma + haemolysate (diluted 1 in 10) + 1/40 M $CaCl_2$	3	24	4	17

Antiheparin Activity.—Lysed erythrocytes show a marked antiheparin activity comparable to the activity of a concentrated platelet suspension, and it is directly proportional to the concentration of haemolysate and not inhibited by an excess. It is poor when 1 in 50 diluted samples are used (Fig. 2). It is stable to temperature (60° for 30 min.) and storage and does not decrease after treatment with $BaSO_4$. The antiheparin effect of haemolysate is rapid and does not increase after incubation with heparinized plasma.

Effect on Other Coagulation Tests.—Haemolysate does not significantly influence the thrombin-fibrinogen reaction, but in the most concentrated samples there is a slight inhibitory action. On the contrary, platelets show a slight but constant accelerating effect (Table V).

TABLE V

EFFECT OF HAEMOLYSATE ON THROMBIN-FIBRINOGEN REACTION

Solution Added	Clotting Times in Seconds (Average of Four Tests) of 0·4 ml. Fibrinogen + 0·1 ml. Thrombin (2 units per ml.)
Saline	16·3
Haemolysate diluted 1 in 2	19
" " 1 " 10	16·5
" " 1 " 50	16·5
Platelet suspension (300,000 per c.mm.)	14·5

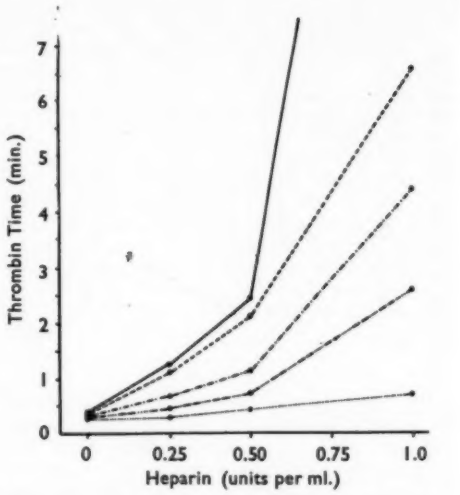

FIG. 2.—The addition of haemolysate can shorten the thrombin time of heparinized plasma. ——— saline. – – – – – haemolysate diluted 1 in 50. –·–·–·–·–·– haemolysate diluted 1 in 10 —···—···— platelet suspension (300,000 per c.mm.). ····· haemolysate diluted 1 in 2.

The clotting time of platelet-poor normal plasma to which Russell's viper venom and $CaCl_2$ were added (" stypven " time) was markedly accelerated by the haemolysate. The accelerating activity depends on the concentration of the added haemolysate and is still present in a dilution of 1 in 1,000. The strongest activity is shown by haemolysate diluted 1 in 10. It does not change when using more concentrated samples (Table VI).

The highest concentrations of haemolysate inhibit the Quick time of platelet-poor normal plasma. A similar inhibiting effect of the less dilute samples of haemolysate was observed in the test for factor VII, which was not shown in the test for factor V activity. Very slight factor V activity was observed in undiluted haemolysate and it is probably due to absorbed proteins that could not be washed off the erythrocytes (Table VI).

The factor VII activity of a normal serum decreases slightly after incubation with excess haemolysate and persists unchanged if incubated with diluted samples (Table VI). No evidence of any inhibitory effect of the haemolysate on brain thromboplastin or plasma thromboplastin was shown in our experiments. No clot-retraction promoting activity was observed in the haemolysate.

TABLE VI
INHIBITORY EFFECT OF EXCESS HAEMOLYSATE ON CLOTTING TESTS

Tests:	Quick Time (sec.)		Stypven Time (sec.)	Factor VII Activity (sec.)	Factor V Activity (sec.)	Test for Factor VII Inhibition (sec.)	
Reagents:	0·1 ml. Brain Thromboplastin +0·1 ml. 0·025 M $CaCl_2$ Added to:		0·1 ml. Platelet-poor Plasma +0·1 ml. Russell Viper Venom (Diluted 1 in 3 in Saline) +0·1 ml. 0·025 M $CaCl_2$	0·1 ml. Seitz-filtered Ox Plasma +0·1 ml. Brain Thromboplastin +0·1 ml. 0·025 M $CaCl_2$	0·1 ml. Oxalated Stored Plasma +0·1 ml. Brain Thromboplastin +0·1 ml. 0·025 M $CaCl_2$	0·1 ml. Normal Serum Diluted 1 in 10 in Buffer, + 0·1 ml. test material Before and After 30 min. Incubation at 37° C. Added to 0·1 ml. Seitz-filtered Ox Plasma +0·1 ml. Brain Thromboplastin +0·1 ml. 0·025 M $CaCl_2$	
Solution (0·1 ml.) Added	0·1 ml. Normal Plasma	0·01 ml. Normal Plasma +0·09 ml. $BaSO_4$-treated Stored Plasma				Before Incubation	After Incubation
Saline	20·7	60	30·5	90	70	60·5	61·7
Haemolysate undiluted ..	—	—	10	—	61	—	—
,, diluted 1 in 2	23	85	10	109	—	58·5	64
,, ,, 1 ,, 5 ..	—	—	10·5	—	—	—	—
,, ,, 1 ,, 10	20	60·5	10·3	86	70	54	55·5
,, ,, 1 ,, 50	20·5	59	13	87	71	59	57
,, ,, 1 ,, 100	—	—	17	—	—	—	—
,, ,, 1 ,, 500	—	—	21	—	—	—	—
,, ,, 1 ,, 1,000	—	—	26·3	—	—	—	—
Platelet suspension (300,000 per c.mm.)	20·5	59	—	83	56	52	50·5
Normal fresh plasma diluted 1 in 10	—	—	—	42	38	—	—

Discussion

It is known that erythrocytes contain a thromboplastic factor, probably of a lipoid nature and with a platelet-like activity. It can be substituted for platelets in the thromboplastin generation test or in the prothrombin utilization test and in assessing thrombin formation. It has been used by Bounameaux (1957b) for the assay of A.H.G. The preparation of a washed platelet suspension takes time (Wolf, 1956) and its activity deteriorates on storage due to clumping of platelets. Haemolysate, on the contrary, is fairly stable and easy to prepare. The use of a haemolysate instead of platelets might therefore be useful in coagulation tests. Our results show that in clotting tests the use of haemolysate to replace platelet suspensions is inconvenient as it makes these tests less sensitive for the detection of A.H.G. deficiencies.

It seems that the highest concentrations of lysed erythrocytes have an inhibitory effect on clotting. Similar results were obtained using concentrated platelet suspensions or lipid extracts from platelets and from brain in thromboplastin generation tests (Larrieu, Caen, and Bernard, 1955; Bounameaux, 1957a) and in prothrombin consumption tests (Wolf, 1956). Wolf believed that the inhibition exerted by excess lipid or platelets was due to a blockage of the reaction between thromboplastin and proconvertin (factor VII) because it does not occur in the " stypven " time. On the other hand, Bounameaux (1957a) found that excess platelets increase the rate of inactivation of plasma thromboplastin. We did not find that haemolysate had any antithromboplastin action after incubation either with brain or plasma thromboplastin. Since inhibition is exerted by concentrated haemolysate on thromboplastin and thrombin generation and on Quick time, while it does not occur in the presence of Russell's viper venom, this action is probably identical with that previously described for excess lipid and platelets. Our results support such a view. It cannot, however, be explained by this theory why inhibition takes place during plasma thromboplastin generation, for which, according to Ackroyd (1956), factor VII should not be required. Slight inhibition by the concentrated haemolysate of the thrombin-fibrinogen reaction was also observed.

We cannot explain whether inhibition from excess haemolysate might be due to a specific antithromboplastic substance, such as Tocantins' lecithin fraction from brain (Tocantins, Carroll, and McBride, 1948), or to a different activity of the same thromboplastic factor at different concentrations. Wolf (1956) and Bounameaux (1957a) believe that gross excess of one clotting factor can cause an inhibitory effect. On the other hand, Barkhan, Newlands, and Wild (1956) have isolated two phospholipids, phosphatidyl-ethanolamine and phosphatidyl-serine, from acetone-dried brain tissue, the former accelerating and the latter inhibiting plasma thromboplastin generation. Therefore, the possibility of the presence in the erythrocytes of various

phospholipids with different accelerating or inhibiting activities cannot be excluded.

Erythrocytes as well as platelets can block the antithrombin activity of heparin. According to Deutsch *et al.* (1955) platelet antiheparin factor is different and separable from the thromboplastic factor by ultracentrifugation. The heparin-neutralizing faculty of red cells behaves like the erythrocyte thromboplastic activity, but it is much less in progressive dilutions of the haemolysate and it is not inhibited by an excess. Because of the different mechanism of the two activities, there is no evidence to assume the existence of two different factors in erythrocytes.

The lipoid factor, isolated from erythrocytes by de Vries, Kettemborg, and van der Pol (1956), was claimed to be devoid of antiheparin activity, but actually it was inactive on the thromboplastin generation test also and it could only shorten the "stypven" time. Therefore, the only evidence following from de Vries' results is that the accelerating effect on "stypven" time is not always associated with thromboplastic activity and cannot be identified with it.

Summary

The activity of lysed human erythrocytes in the various stages of the clotting process was investigated. Some characteristics of erythrocyte thromboplastic activity were defined by the use of different tests. The mechanism of the inhibitory effect of concentrated haemolysate was investigated. It may be due to an inhibition of factor VII. Slight antithrombin activity was also observed. It was found that the use of haemolysate as a substitute for platelets makes clotting tests less sensitive to detect A.H.G. deficiencies.

A stable antiheparin activity was detected in erythrocytes.

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FACTOR VII AND HEPARIN IN THROMBOSIS

BY

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(RECEIVED FOR PUBLICATION AUGUST 25, 1958)

Factor VII is the term used by Koller, Loeliger, and Duckert (1951) for the accelerator complex of prothrombin conversion present in both plasma and serum which is markedly reduced by treatment with drugs of the dicoumarin group.

It has been shown by Poller (1954 and 1956a) that there is a significant reduction in the anticoagulant action of heparin on the plasma clotting times of patients with recent thrombo-embolic disease. It has also been shown by Poller (1957) that there was a significant increase in plasma factor VII activity in such patients at the same stage of their illness.

The aim of the present investigation was to determine whether there was any association between the increase in factor VII activity and the "heparin resistance" of these cases. The problem is of particular interest because both factor VII and heparin are of established importance in maintaining the normal balance of coagulation.

Method of Study

To assess the effect of a factor VII preparation on heparin plasma clotting times, increasing volumes of the factor VII preparation were added to plasma clotting mixtures containing a standard amount of the heparin anticoagulant. In a second series of tubes serum rich in factor VII was substituted for the factor VII preparation, and in a third normal saline was used as a control.

Reagents

Platelet-rich Plasma.—Fresh oxalated plasma was obtained by spinning venous blood collected without undue venostasis into a siliconed syringe and diluted 1 in 10 with 1% potassium oxalate.

Heparin.—Standard solution of heparin 10 units/ml. (Evans).

Calcium Chloride (0.25%).

Stored Serum.—This was obtained from normal venous blood allowed to clot at 37° C., separated, and the serum stored at room temperature for 24 hours. The factor VII activity of the resulting serum in restoring the prothrombin defect in "dindevan" plasma was approximately twice that of the original plasma, volume for volume.

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Factor VII Preparation

This was prepared from normal stored serum by adsorption and elution using the method described by Biggs and Macfarlane (1953). The resulting factor VII activity was many times that of the original serum, volume for volume.

Procedure

Three rows of six tubes were assembled in a 37° C. water-bath. To each tube 0.5 ml. of plasma and 0.1 ml. (1 unit) of heparin were added. To the first row of tubes volumes of physiological saline were added from 0.1 ml. in the second to 0.5 ml. in the sixth.

To the second row similar volumes of normal stored serum were added, and to the third row the same increasing volumes of the factor VII solution.

To each tube in the first row 0.5 ml. of 0.25% calcium chloride was added at staggered 15-second intervals, and the clotting time noted. The procedure was continued at 15-second intervals through all three rows of tubes. (The clotting times were performed simultaneously to reduce the effect of storage *in vitro* on the results.)

The experiment was performed three times and the results expressed as an average for the three investigations.

Results

The results were similar on all three occasions, and the mean values are given in Fig. 1. The clotting time is markedly shortened by the addition of the factor VII solution, and with volumes of 0.3 ml. and more the anticoagulant effect of the heparin appears to have been completely neutralized. The results in tubes 4, 5, and 6 are similar to those obtained in a series of normal plasma clotting times using the same technique but without the addition of heparin and factor VII.

Serum rich in factor VII activity has a similar but less marked antagonism to heparin.

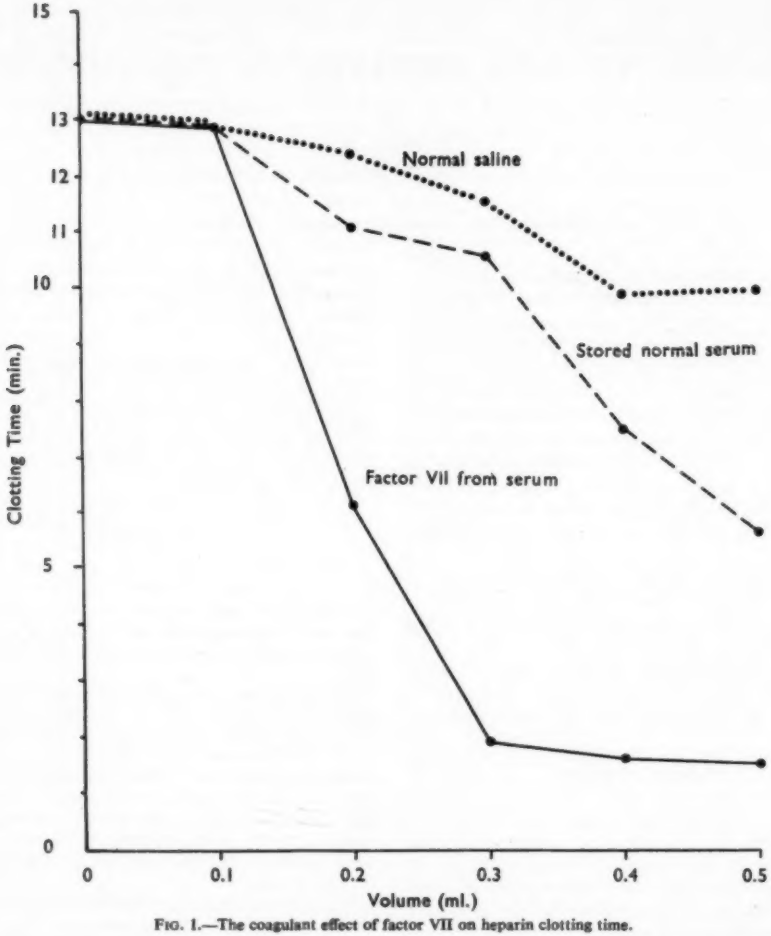

FIG. 1.—The coagulant effect of factor VII on heparin clotting time.

The slight effect produced by saline may have been due to simple dilution of the calcium chloride to an optimum strength for coagulation.

Effect on Thrombin Generation. — Further confirmation of the marked antagonism between a serum factor VII preparation and heparin was obtained from the rate of thrombin generation in plasma clotting in the presence of heparin. The results are given in Fig. 2. Line A shows the slow rate of thrombin generation in heparin plasma clotting times. (The results here are the mean of three investigations.) In lines B, C, D, thrombin generation in three subjects is shown, using an identical technique except for the addition of 0.5 ml. of the factor VII solution to the heparin plasma clotting time. The anticoagulant action of heparin appears to have been completely neutralized by the factor VII solution, the pattern resembling thrombin generation in simple plasma clotting without heparin.

Discussion

It may be seen from the above investigations that the factor VII-rich extracts prepared by adsorption and elution of serum possessed a marked antagonistic action to heparin in plasma clotting and in thrombin generation.

The antiheparin activity may be due to one of three other serum factors adsorbed and eluted with factor VII. Van Creveld and Paulssen (1951

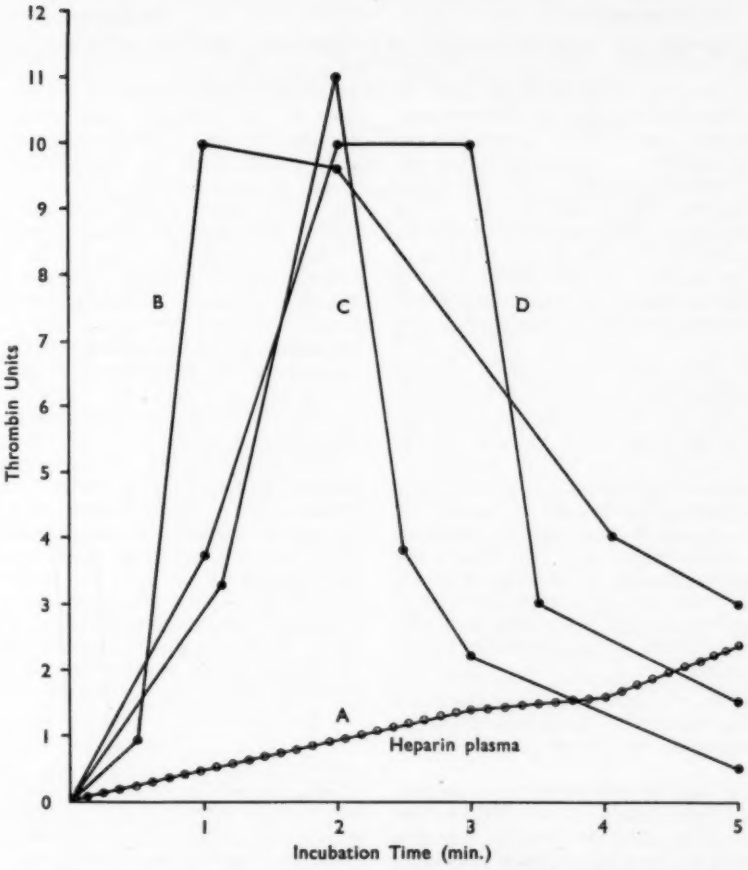

FIG. 2.—The coagulant effect of factor VII preparation (0.5 ml.) on heparin plasma clotting time in three normal adults (B,C,D).

and 1952) described an antiheparin platelet factor. Serum extracts are also known to contain Christmas factor and possibly factor X (Duckert, Flückiger and Koller, 1954) which may have been responsible.

The effects of a factor VII preparation prepared from recalcified platelet-deficient plasma collected into siliconed glassware were found to be equally marked. It thus appears unlikely that the effect is due to the antiheparin factor of platelets.

The antiheparin activity of sera from eight severe cases of Christmas disease was significant using the heparin plasma clotting technique. The effect was less than in a normal group, but the results were not significantly different. Christmas factor therefore does not appear to be the important serum heparin antagonist. Active antiheparin factor VII preparations were made from Christmas serum.

Factor X is unstable at room temperature and is markedly decreased in liver disease (Duckert *et al.*, 1954). Serum was obtained from cases of parenchymatous liver disease and was stored at room temperature for 48 hours, and showed impaired activity in thromboplastin generation. The resulting serum extract, however, showed marked antiheparin activity in plasma clotting. It seems very unlikely, therefore, that the effect could be due to factor X adsorbed and eluted with factor VII.

Conclusions

The demonstration of marked antagonism between factor VII and heparin provides a link between two previous observations in cases of thrombosis. The first was that the anticoagulant action of heparin is less marked in the presence of recent thrombosis, a fact established by De Takats by injection *in vivo* (1943a and b) and by work *in vitro* by Waugh and Ruddick (1944a and b), by Rosenthal and Weaver (1952), and by Poller (1954, 1955, 1956). The second observation made by Poller (1957) was that in a group of patients suffering from recent thrombo-embolic disorders there was significantly increased plasma factor VII activity.

The exact relationship of these findings to abnormal intravascular clotting is not clearly defined. There are two possible explanations. The first is that increased factor VII activity may be a causative agent in the production of thrombosis, neutralizing the natural anticoagulant heparin and upsetting the balance of coagulation. This role is suggested by the experimental work of Wessler (1954, 1955), who produced rapid thrombosis in animals by the infusion of serum rich in factor VII activity. Indirect evidence to support this theory is also provided by Koller *et al.* (1952), by Ciulla and Santoni (1954), and by Alexander, Meyers, Goldstein, Gurewich, and Grinspoon (1954), who found increased factor VII activity during pregnancy which they presumed to be associated with the "hypercoagulability of pregnancy."

The second possible explanation is simpler, namely, that the increased factor VII activity results from serum factors released into the circulation from the thrombus (serum containing more factor VII than plasma). Indirect support for this may be deduced from the fact that the shortest heparin plasma clotting times occurred in major vessel occlusion, e.g., thrombophlebitis group, mean 4.6 minutes with a standard deviation (S.D.) 1.04, whereas the coronary thrombosis group gave a mean of 6.25 minutes (S.D. 2.1) (Poller, 1956a).

Whatever the exact relationship of these changes the demonstration of increased factor VII activity and the role of factor VII in neutralizing the anticoagulant heparin provide a firm basis for the administration of anticoagulant drugs to restore the disturbed balance of coagulation.

Summary

Serum extracts rich in factor VII activity prepared by adsorption and elution markedly reduce the anticoagulant action of heparin on plasma clotting and thrombin generation. This fact provides a link between the two previous observations made by the author in cases of recent thrombo-embolic disorders, namely, that there is resistance to the anticoagulant action of heparin and increased plasma factor VII activity.

There is indirect evidence to support two possible theories of the relationship to thrombosis. The first possibility is that factor VII plays an important role in the actual causation of thrombosis by its antiheparin action. The second explanation is that increased plasma activity results simply from the absorption into the circulation of factor VII liberated from the thrombus.

The demonstration of the neutralization of natural anticoagulant by factor VII provides a firm basis for the administration of the dicoumarin type drugs in these conditions, of which the main function is to depress the raised level of factor VII activity.

I should like to thank Dr. M. C. G. Israels and Dr. R. G. Macfarlane for advice on this work, and the Department of Haematology, Manchester Royal Infirmary, for batches of Christmas serum.

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A RAPID CYTOLOGICAL METHOD FOR THE DIAGNOSIS OF MEASLES

BY

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(RECEIVED FOR PUBLICATION APRIL 27, 1959)

The fact that the laboratory diagnosis of virus infections is a lengthy process is the constant complaint of our clinical colleagues. The purpose of this paper is to call attention to the method of exfoliative cytology as a rapid method for the diagnosis of measles. It has been established that the characteristic pathological feature of measles is the appearance of large multinucleated giant cells, particularly in areas of lymphoid tissue, as first described by Denton (1925). The work of Tompkins and Macaulay (1955) has demonstrated that similar syncytial giant cells are present in sputum and may be recovered by aspirating mucus from the back of the nose. It is striking that measles virus also produces syncytial giant cell masses when growing in tissue culture on epithelial type cells (Enders and Peebles, 1954). The particular value of this method of diagnosis is that the giant cells are present during the catarrhal stage, disappearing rapidly with the onset of the rash. It is easy to obtain material which can be rapidly stained and examined in the laboratory, enabling a diagnosis to be made in a few hours. This may be valuable with suspected measles, for example, in a children's ward. Similar giant cells do not seem to occur in other diseases, although somewhat similar cells are seen in nasal mucus of ferrets infected with canine distemper virus. Also Done (1958) has recently described a similar method for the diagnosis of inclusion body rhinitis of swine. He examined nasal secretions of pigs using Giemsa or methylene blue staining. The pathognomonic cells contain intranuclear inclusions. The giant cells seen in herpes simplex and varicella are quite different from those seen in measles, having fewer nuclei, which often overlap and contain less cytoplasm.

Method

Aspiration of Nasal Mucus.—At first nasal mucus was collected by the method described by Tompkins and Macaulay (1955). This involved pushing a glass pipette along the floor of the nose and aspirating mucus from the posterior turbinate region. Because of a natural fear of damaging the child insufficient mucus for examination was frequently obtained. Later a better method of obtaining sputum from children, which was described by Auger (1939), was used. In this method a polythene tube attached to a syringe (preferably at least of 20 ml. capacity) is inserted into the back of the nose and then suction is applied. In order to obtain a second suction stroke a small hole can be cut in the side of the tubing which enables air to be expelled without dislodging the mucus. During suction the hole is occluded.

Staining. — Papanicolaou's method was used following in general the technique described by the staff of the Vincent Memorial Hospital (1950), using smears fixed immediately in ether-alcohol mixture. (This technique was taught by the staff of the General Hospital, Newcastle upon Tyne, through the courtesy of Dr. B. E. Tomlinson.) Tompkins and Macaulay report that methylene blue staining of air-dried films is adequate, and working with Dr. Morley, of Ilesha, Nigeria, it has been found that very good results can be obtained using Giemsa staining of air-dried smears.

Results

In a florid case in the catarrhal stage it is easy to obtain mucus which is full of the characteristic giant cell masses. A low-power view of the appearances seen is shown in Fig. 1. If material is taken from the front of the nose or later on in the disease when the rash has appeared large necrotic masses are often present in which it is impossible to decipher a giant cell. Material collected earlier (up to five days before the onset of the rash) during the catarrhal stage of the disease from the posterior turbinate region contains easily recognizable giant cells. Higher-power views of these cells are shown in Figs. 2 and 3. It seems probable that these cells are derived from fusion of ciliated mucosal cells that have become desquamated. Tompkins and Macaulay (1955) describe having seen cilia on the edge of the cells, but we have not observed this. Fig. 4, however, shows cells coming together and we think that these go on to form the giant cell masses by dissolution of the cell boundaries.

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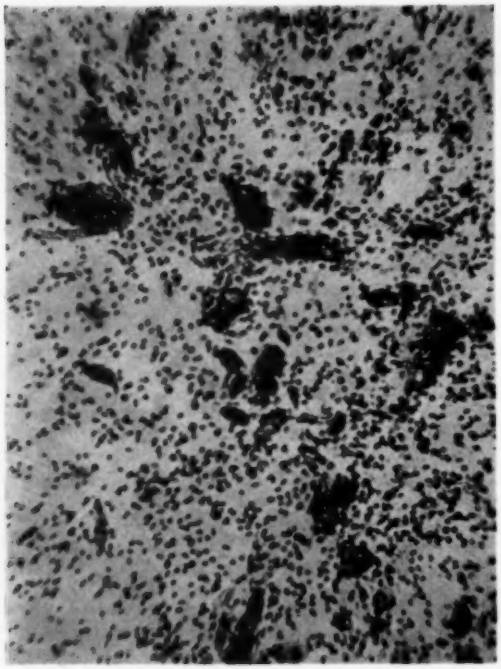

FIG. 1.—Low-power view of measles giant cells obtained on the day of onset of the rash (Papanicolaou × 100).

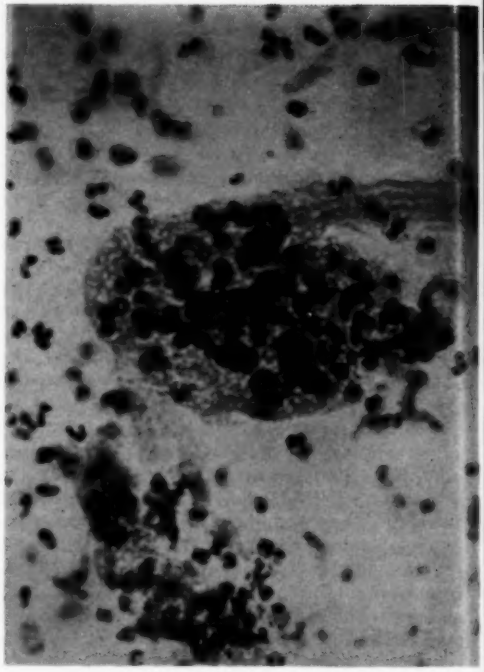

FIG. 2.—High-power view of a cell from Fig. 1 (Papanicolaou × 500).

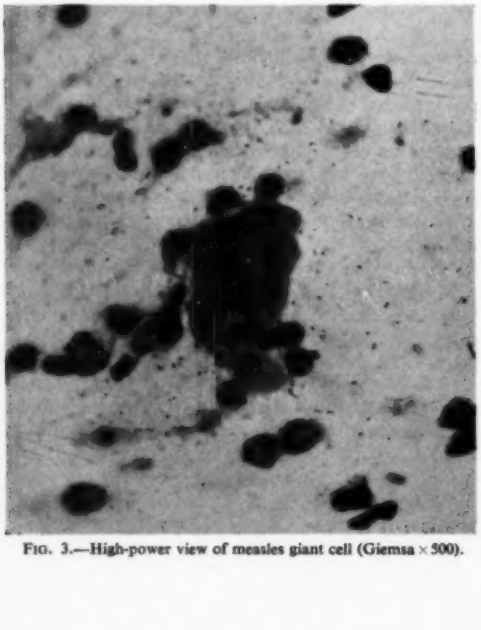

FIG. 3.—High-power view of measles giant cell (Giemsa × 500).

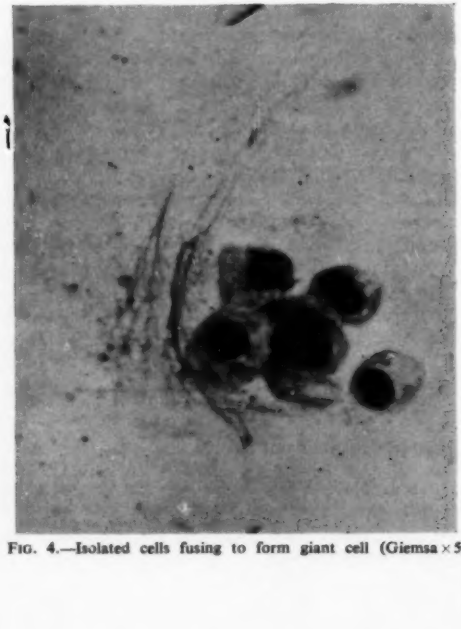

FIG. 4.—Isolated cells fusing to form giant cell (Giemsa × 500).

In the winter of 1956–57 there was an epidemic of measles in Newcastle upon Tyne, as elsewhere in England, and during the spring of 1957 this method of diagnosis was put to the test with the co-operation of the general practitioners in the city. In all 32 smears were examined. When the general practitioner visited a case of suspected measles in the prodromal stage he took material from the child's nose, fixed it in 50–50 ether alcohol and sent it to the laboratory. When the result was reported to the doctor he in turn reported whether a measles rash had developed. During this time mucus was collected with a glass pipette which did not prove very satisfactory, and this almost certainly accounts for the rather high proportion of false negatives. As shown in the Table, it will be seen that 13 patients who developed measles showed giant cells, whereas 10 patients who had measles failed to show giant cells. On the other hand there were no false positives ; all the nine patients who did not develop a rash did not show giant cells. Similar giant cells have not been seen in other children with catarrhal symptoms not due to measles. In many hundreds of sputum specimens examined for the diagnosis of carcinoma of the bronchus the appearances seen in measles have not been observed (Dr. B. E. Tomlinson, personal communication).

TABLE
EXAMINATION OF NASAL SMEARS FROM CHILDREN WITH CATARRHAL SYMPTOMS FOR MEASLES GIANT CELLS

Number of Patients	Giant Cells Present	Measles Rash
13	+	+
10*	−	+
9	−	−

* In at least five of these the specimen was not adequate for examination.

Discussion

Giant cells are the characteristic pathological feature of measles virus infection both in the intact animal (man or monkey) and in tissue culture. Recently Sherman and Ruckle (1958) described and illustrated these changes. There are two sorts of giant cells seen in measles.

1. Epithelial Giant Cell or Syncytium.—This is most common in the respiratory tract, but sometimes occurs on other epithelial surfaces, for example, the bladder. It is these cells desquamated from the respiratory tract which enable a cytological diagnosis of measles to be made. They are not known in other conditions with the exception of Hecht's giant cell pneumonia which is probably in reality due to measles virus. Similar giant cells have been seen in canine and mink distemper (Pinkerton *et al.*, 1945). These epithelial type giant cells also occur in tissue cultures infected with measles virus (Enders and Peebles, 1954) and contain eosinophilic intranuclear inclusions. It is generally acknowledged that these cyncytial masses arise by fusion of cells.

2. Reticulo-endothelial Type Giant or Warthin-Finkeldey Cell.—These cells are occasionally encountered in the appendix removed from prodromal measles cases and may arise by fusion or by phagocytosis. Intranuclear inclusions are rarely seen in them in tissue sections, but Sherman and Ruckle (1958) reported that intranuclear and intracytoplasmic inclusions were present in cells cultured from measles-infected spleen or lymph nodes.

So far as is known the presence of these giant cells is specific for measles infection. Unfortunately in this study it did not prove possible to examine large numbers of control children in order to establish this definitely. However, giant cells were not seen in patients not suffering from measles. Similarly Tompkins and Macaulay (1955) report that giant cells were not seen in other upper respiratory infections or allergic rhinitis. Multinucleated giant cells are characteristic of some other virus infections, for example, herpes simplex and varicella. The giant cell seen in these diseases is different, having fewer nuclei and less cytoplasm, and can be obtained by scraping the floor of a chickenpox vesicle. If seen a distinction can be made between herpes simplex and varicella (showing giant cells) and smallpox and vaccinia (not showing giant cells), for example.

Although measles is not often a difficult diagnostic problem there are occasions, especially in children's hospitals, where it would be most valuable to be able to make a diagnosis in the catarrhal stage. It is our object to call attention to a simple and rapid method which may well enable this to be achieved.

Our thanks are due to general practitioners in Newcastle upon Tyne who co-operated in this study, and to Mr. S. A. Keeble for the photomicrographs.

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GASTRIC PROTEOLYSIS IN DISEASE

2. THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE AND GASTRIC MUCOSAL EXTRACTS FROM PATIENTS WITH CHRONIC GASTRIC AND DUODENAL ULCER

BY

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(RECEIVED FOR PUBLICATION JANUARY 30, 1959)

The secretion of pepsin in the gastric juice of patients with peptic ulcer has been the subject of much investigation. Early work was carried out mainly on resting juices or on the aspirated products of an Ewald meal (Willcox, 1908; Boas, 1925; Hirsch-Mamroth and Rindfleisch, 1925; Polland and Bloomfield, 1930; Vanzant, Osterberg, Alvarez, and Rivers, 1933; Mullins and Flood, 1935), and appeared to establish that in both gastric and duodenal ulcer, but more strikingly so in the latter, the pepsin concentration of the gastric juice is increased. The total output of pepsin in response to a given stimulus is further increased because a greater volume of juice is secreted. Ihre (1938) found, however, in both chronic duodenal and gastric ulcer that the pepsin concentrations of the fasting and histamine secretions were normal and that only after insulin hypoglycaemia were they raised. Le Veen and Hallinger (1947) presented evidence that the absolute peptic activity of the fasting gastric juice in peptic ulcer was normal and only appeared raised because of the lower *p*H at which the fasting juice is usually secreted in this disease. Chinn, Book, and Beams (1951) also were unable to find any significant difference in the pepsin concentration of nocturnal or insulin juices between patients with duodenal ulcer and normal controls. Janowitz and Hollander (1951, 1952) determined the total pepsin content of the basal gastric secretion (" in the absence of all intentional and avoidable stimuli ") over periods of one hour, and found that in duodenal ulcer the average basal output of pepsin is up to three times that of normal controls, whereas in gastric ulcer it is not significantly different from normal. Whilst, therefore, it is doubtful if the actual concentration of pepsin is increased, it seems established that, in duodenal ulcer at any rate, because of the increased volume of gastric secretion, the total output of pepsin by the mucous membrane both at rest and in response to a given stimulus is usually greater than in normal subjects.

The underlying assumption in these investigations has been that the gastric mucous membrane of patients with peptic ulcer secretes normal pepsin. Proteolytic activity has been determined only near *p*H 2. The possibility that modified pepsin, or enzymes different from pepsin, may be present has not been considered. An investigation of this possibility is now described.

Materials and Methods

The materials and methods used have been described previously (Taylor, 1956, 1957, 1959a). Portions of stomach from patients undergoing partial gastrectomy were cooled immediately to below 5° C. The mucous membrane was dissected away from the muscle coat, cut into small pieces, and extracted in a blender with 2% (w/v) sodium chloride. The extract was then centrifuged to remove debris and stored at −5° C. The separation of fundic and pyloric mucosa has already been defined (Taylor, 1959c).

Results

Gastric Juice.—Three patients with duodenal ulcer and one with gastric ulcer exhibited *p*H activity curves with two maxima when their histamine-stimulated gastric juices digested plasma protein below *p*H 5 (Table I). The maxima occurred between *p*H 1.9 and 2.4, and 3.3 and 3.6, and are thus similar to the two maxima which occurred with the gastric juice of 15 of 17 normal subjects under the same experimental conditions (Taylor, 1959a).

The remaining seven patients each exhibited a different *p*H activity curve for the digestion of plasma protein or plasma albumin fraction V, in which three maxima occurred below *p*H 5. The *p*H ranges of these maxima were 1.5 to 2.1, 2.5 to 3.1, and 3.3 to 3.8 (Table I). The *p*H activity curve for the digestion of egg albumen was

TABLE I

*p*H MAXIMA FOR DIGESTION OF PROTEINS BELOW *p*H 5 BY HISTAMINE-STIMULATED GASTRIC JUICES OF PATIENTS WITH PEPTIC ULCER

Patient	Substrate	*p*H 1·5 to 2·4		*p*H 2·5 to 3·2		*p*H 3·3 to 4·0	
		Maximum *p*H	Amino-acid N Released (mg.)	Maximum *p*H	Amino-acid N Released (mg.)	Maximum *p*H	Amino-acid Released (mg.)
1	Plasma protein ..	1·9	0·60	—	—	3·6	0·45
2		2·2	0·55	—	—	3·3	0·50
3		2·1	0·62	—	—	3·3	0·60
*4		2·4	0·78	—	—	3·3	0·83
5		2·1	0·46	2·7	0·41	3·7	0·41
6		1·8	0·45	2·9	0·35	3·7	0·36
7		1·8	0·46	3·0	0·48	3·8	0·45
8		1·5	0·35	2·5	0·40	3·3	0·38
*9		1·6	0·66	2·6	0·57	3·5	0·63
*10		1·9	0·57	2·6	0·64	3·4	0·64
11	Bovine plasma albumin fraction V	2·0	0·65	3·1	0·67	3·8	0·72
5	Egg albumen	1·5	0·31	2·6	0·22	Declining activity	
6		1·6	0·24	2·8	0·14	,,	,,
8		1·6	0·31	3·1	0·17	,,	,,

* Patients with gastric ulcer; remainder had duodenal ulcer.

In the tables and figures the amounts of amino-acid nitrogen released are those in the whole digest, which consisted of 3 to 5 ml. of buffer, 0·5 to 1 ml. of gastric juice or extract, and 1 ml. of plasma or of 5% (w/v) albumin solution. Temperature 37°, time 3 hr.

TABLE II

*p*H MAXIMA FOR DIGESTION OF PROTEINS BELOW *p*H 5 BY GASTRIC MUCOSAL EXTRACTS OF PATIENTS WITH PEPTIC ULCER

Patient	Extract	Substrate	*p*H 1·5 to 2·4		*p*H 2·5 to 3·2		*p*H 3·3 to 4·0	
			Maximum *p*H	Amino-acid N Released (mg.)	Maximum *p*H	Amino-acid N Released (mg.)	Maximum *p*H	Amino-acid N Released (mg.)
*9	Whole mucosal	Plasma protein	1·8	0·65	2·6	0·63	3·5	0·69
12		Human serum albumin	1·5	0·63	2·6	0·69	3·7	0·74
13			2·4	0·63	—	—	3·6	0·73
8	Fundic	Plasma protein	2·2	0·70	2·9	0·77	3·5	0·63
*10			1·7	0·34	2·7	0·36	3·6	0·24
11			2·2	0·90	2·9	0·90	3·6	0·85
14			2·1	0·61	3·0	0·68	3·9	0·70
15			2·0	0·64	3·0	0·63	3·9	0·46
16			1·6	0·63	2·5	0·61	3·3	0·55
17			2·1	0·70	3·1	0·82	3·9	0·64
18			1·9	0·77	3·2	0·85	3·9	0·82
19			1·8	0·63	—	—	3·5	0·72
8		Human serum albumin	2·3	0·76	—	—	3·6	0·87
20		Bovine plasma albumin fraction V	2·1	0·30	2·9	0·27	3·8	0·25
21			1·5	0·63	2·5	0·63	3·4	0·84
22			Plateau		Plateau		3·6	0·80
23			,,		,,		3·5	0·91
8		Egg albumen	1·5	0·30	2·5	0·22	Declining activity	
11			1·7	0·41	3·1	0·20	,,	,,
17	Lesser curve	Plasma protein	1·9	0·62	—	—	3·6	0·50
18			1·9	0·18	3·1	0·20	3·9	0·20
8	Pyloric	Plasma protein	1·9	0·30	2·5	0·49	3·7	0·47
*10			2·1	0·17	2·8	0·22	Declining activity	
14			1·7	0·56	2·5	0·55	3·4	0·50
15			1·7	0·16	2·8	0·25	Declining activity	
16			2·1	0·20	—	—	3·4	0·28
17			1·8	0·12	3·1	0·13	Declining activity	
18			1·7	0·25	2·8	0·18	3·8	0·28
24			2·1	0·70	3·0	0·20	Declining activity	
25			2·0	0·20	3·2	0·25	,,	,,
8		Egg albumen	1·5	0·15	3·0	0·12	Declining activity	
11			1·4 and 2·2	0·09 0·08	Declining activity		,,	,,

* Patients with gastric ulcer; the remainder had duodenal ulcer.

determined in three of these patients, but only two maxima, at *p*Hs 1.5 to 1.6 and 2.6 to 3.1, were observed. The gastric juice of one patient (No. 11) failed to digest clupeine sulphate.

Whole Gastric Mucosal Extracts. — Whole gastric mucosal extracts from one patient with gastric ulcer and two with duodenal ulcer have been incubated with plasma protein or human serum abumin. One patient showed a normal *p*H activity curve with two maxima, but two gave curves with three maxima (Table II).

Fundic Mucosal Extracts. — Fundic mucosal extracts from nine patients with duodenal ulcer and one with gastric ulcer digested plasma protein or plasma albumin fraction V with three maxima below *p*H 5 (Fig. 1, Table II). The three maxima occurred within the ranges, *p*H 1.6 to 2.2, 2.5 to 3.2, and 3.3 to 3.9. One patient (No. 19) exhibited a normal *p*H activity curve with only two maxima, and two (Nos. 22 and 23) exhibited abnormal curves with a single maximum at *p*H 3.5 to 3.6. These latter curves were, however, not symmetrical ; there was high activity at *p*H 2, but no significant maxima could be distinguished.

Of those patients showing three maxima, the highest proteolytic activity occurred in four at the middle maximum, and in a fifth patient the lowest and middle maxima exhibited equal proteolytic activity. In two patients a strip of mucosa 1 in. wide, taken from the length of the lesser curve of the stomach but excluding the 2 in. nearest to the pyloro-duodenal junction, was extracted separately. One such extract digested plasma protein with three maxima below *p*H 5, but the other showed only two maxima. One extract (No. 8) which digested plasma with three maxima digested

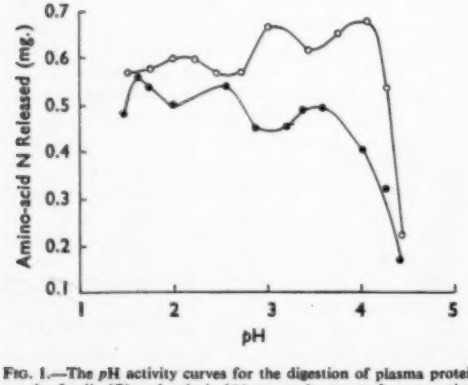

FIG. 1.—The *p*H activity curves for the digestion of plasma protein by fundic (O) and pyloric (●) mucosal extracts from a patient with duodenal ulcer, 37° C., 3 hr.

serum albumin with two. The experimental readings showed a third maximum, but this was not significant by the criteria that are applied (Taylor, 1956, 1959a). Two extracts which digested plasma with three maxima digested egg albumen with only two.

Pyloric Mucosal Extracts. — Pyloric mucosal extracts from eight patients with duodenal ulcer and one with gastric ulcer have been incubated with plasma protein (Table II). Of these extracts three gave *p*H activity curves with three maxima at *p*H 1.7 to 1.9, 2.5 to 2.8, and 3.4 to 3.8 (Fig. 1). Six gave curves with two maxima occurring in the *p*H ranges 1.7 to 2.1 and 2.8 to 3.4. Two extracts, one of which had digested plasma protein with three maxima, digested egg albumen with two maxima at *p*H 1.4 to 1.5 and 2.2 to 3.0.

Proteolytic Activity at *p*H 5 to 8.—Proteolytic activity between *p*H 5 and 8 has been investigated in seven patients with duodenal ulcer. The whole mucosal extract of one was inactive when incubated with plasma, but that of a second patient digested human serum albumin weakly with a maximum at *p*H 7.2. Four fundic extracts were incubated with plasma ; two were inactive, one was active over the range *p*H 6.4 to 7.4, but no maximum could be detected, and the fourth was weakly active with a maximum at *p*H 7.2. The pyloric extracts of two of these patients digested plasma protein weakly at *p*H 6.4 to 7.4, but no maxima could be distinguished. The pyloric extract of a seventh patient was unable to hydrolyse crystalline bovine plasma albumin. Thus proteolytic activity was present in three of six extracts which contained fundic mucosa, and in three of five containing pyloric mucosa.

Hydrolysis of Benzyloxycarbonyl-L-glutamyl-L-tyrosine.—This compound is a specific substrate for pepsin and is hydrolysed with a *p*H maximum near 4.0 (Fruton and Bergmann, 1939). Normal gastric juice, although digesting most proteins with two maxima below *p*H 5, digests this substrate with only one maximum at *p*H 4.0 (Taylor, 1959a). Histamine gastric juice from two patients with peptic ulcer and a fundic mucosal extract from a third have been incubated with the substrate for 21 hours. Although the juices and extract each digested plasma protein with three maxima below *p*H 5, they hydrolysed the peptide with only one maximum at *p*H 3.6 to 4.0 (Fig. 2), and thus did not differ from normal gastric juice. This result indicates that a similar active site on pepsin is present in normal subjects and in patients with peptic ulcer, but offers no explanation of the origin of the third maximum.

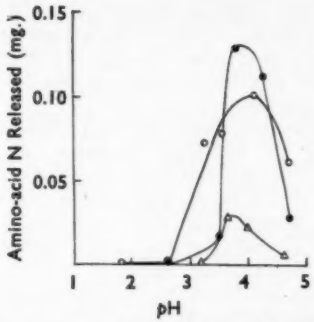

FIG. 2.—The *p*H activity curves for hydrolysis of benzyloxycarbonyl-L-glutamyl-L-tyrosine by gastric juice from a patient with gastric ulcer (△), a patient with duodenal ulcer (●), and by a fundic mucosal extract from a patient with duodenal ulcer (○), 37° C., 3 hr.

Salt Fractionation of Gastric Juice.—In an attempt to separate enzymes responsible for the three proteolytic *p*H maxima that have been observed, gastric juice from patients with peptic ulcer has been subjected to fractionation with ammonium sulphate, sodium chloride, and magnesium sulphate. As was the case with normal gastric juice (Taylor, 1959a), consistent results were difficult to achieve and, as fractionation proceeded, activity was lost. The distinction between maxima and minima was thus blurred, leading to curves with a plateau. It seemed that no separation of one or other peak was taking place, and it was certainly never possible to achieve concentration of the middle peak alone.

Discussion

The most obvious difference between the proteolytic *p*H activity curves of patients with gastric or duodenal ulcer and those of normal subjects is the presence of three maxima in many of the former and the only occasional presence of three maxima in the latter. In peptic ulcer, three maxima were found for the proteolysis of plasma protein in six of 10 samples of histamine juice, in all but one of the fundic extracts, and in three of the nine pyloric extracts examined. This contrasts with normal gastric juice, which exhibited three maxima only in two of 17 subjects (Taylor, 1959a), and with the fundic extracts of five normal subjects and the pyloric extracts of four, all of which digested plasma protein with only two *p*H maxima (Taylor, 1959c).

This difference of gastric proteolytic pattern may be demonstrated in another way. Seventeen normal gastric juices acting on plasma protein exhibited a fall of activity, measured from whichever of the maxima at *p*H 1.7 to 2.4 and 3.3 to 4.0 was the less active to the minimum point in the *p*H range 2.5 to 3.2, of 5% to 34%, with a mean of 13%. In 10 patients with peptic ulcer the fall varied from 3% to 16% (mean 7.7%) and was significantly lower ($t=2.068$, $P<0.05$). There is thus in peptic ulcer a filling-in of the minimum between the two normal maxima, which in about two-thirds of the patients takes the form of a third maximum. In about one-third of the patients this maximum at *p*H 2.5 to 3.1 becomes the most active point of the whole *p*H activity curve.

The curves for egg albumen require special comment. Five of seven normal subjects, whose gastric juice digested other substrates with two maxima, digested egg albumen with only one maximum at *p*H 1.5 to 1.8 (Taylor, 1959a). The two remaining subjects, who had shown three maxima for the digestion of other substrates, digested egg albumen with two maxima, the second occurring at *p*H 2.5 to 2.8. In patients with peptic ulcer, however, all the histamine juices, fundic extracts, and pyloric extracts that have been examined digest egg albumen with two maxima and other substrates with three. One possible explanation of these results might therefore be that there is in the normal population a group of subjects whose gastric juice digests most substrates with three maxima but egg albumen with two, and that peptic ulceration occurs more frequently in this group than in the remainder of the population.

The proteolytic activity of fundic and pyloric extracts from the same patient can be directly compared. In five of seven patients the *p*H maximum in the range 1.6 to 2.4 occurred at higher *p*H values in fundic extracts than in pyloric extracts. The reverse was, however, true in the two remaining cases. The three pyloric extracts with triple-peaked *p*H activity curves also showed *p*H maxima which were generally at lower *p*H values than the corresponding maxima of the fundic extracts. A similar tendency for pyloric extracts to exert maxima at lower *p*H values than fundic extracts has been described in normal subjects (Taylor, 1959c).

The observations upon proteolytic activity at *p*H 5 to 8 in patients with peptic ulcer resembled those of normal subjects in that some patients showed weak activity but others showed none, and that occasional maxima occurred at *p*H 7.2. The chief, and anticipated, difference from normal subjects was the absence of the second peak at

*p*H 7.6 to 7.9 which is thought to arise from salivary contamination (Taylor, 1959d).

Three hypotheses may be advanced to explain the occurrence of *p*H activity curves with three maxima below *p*H 5. First, there might be formation in the mucosa and secretion into the gastric juice of a differing proportion of normal fundic and pyloric pepsins (Taylor, 1959c). Since the former enzyme exhibits *p*H maxima at *p*H 1.8 to 2.4 and 3.3 to 4.0, and the latter at *p*H 1.5 to 1.9 and 2.5 to 3.2, a mixture of both could give rise to a curve with maxima in the required positions. The chief difficulty about this hypothesis is that in such a mixture the maximum at *p*H 1.8 to 2.4 ought to be quantitatively higher than the others, but only in four patients was this so. It is also difficult on this hypothesis to explain the observations made upon egg albumen, for if normal gastric juice digests egg albumen with only a single maximum and yet contains a mixture of two types of pepsin, it would seem that other mixtures of the two enzymes should also yield only one maximum and not two. A further difficulty arises over those pyloric extracts which exhibit a new third maximum at *p*H 3.3 to 3.9. In these patients it must also be supposed that the pyloric mucosa contains more of the fundic type cells.

The second hypothesis is that, in addition to the normal pepsins, a new enzyme is formed and secreted which exerts maximal activity at *p*H 2.5 to 3.2. Such an explanation would be compatible with the finding that activity over this *p*H range may exceed that at *p*H 1.6 to 2.4 and 3.3 to 4.0, and it would account for the observations on egg albumen. It would not account for the third maximum at *p*H 3.3 to 4.0 which occurs in some of the pyloric extracts, unless the hypothesis were extended to include a new pyloric enzyme or the presence in the pylorus of fundic type cells. Attempts to isolate the postulated enzyme by salt fractionation have so far been unsuccessful.

The third hypothesis is that abnormal pepsins are formed and secreted in patients with peptic ulcer which will each digest proteins with three instead of two maxima below *p*H 5. One such enzyme might be produced by fundic cells and a second by pyloric cells. This third hypothesis is not at variance with any of the observations so far made. There is evidence that the double maximal *p*H activity curves which occur in normal subjects arise from the presence in the pepsin molecule of two active centres (Taylor, 1959b). If abnormal pepsins exhibiting three maxima do occur, the acquisition of a third type of active centre would seem a possible explanation of their action. The ability to synthesize the third site for an abnormal fundic pepsin already exists in normal pyloric mucous membrane and the third site for an abnormal pyloric pepsin in normal fundic mucous membrane.

The available evidence does not allow any final distinction to be made between the three hypotheses, but the first seems the least likely. If either the second or third hypothesis proves to be correct it would mean that in peptic ulcer an abnormal or pathological gastric proteinase is being elaborated and secreted. Evidence has already been presented that in pernicious anaemia such a phenomenon may occur (Taylor, 1959e).

Summary

The proteolytic *p*H activity curves of the gastric juice and/or gastric mucosal extracts of 22 patients with duodenal ulcer and three with gastric ulcer have been investigated. No differences of proteolytic activity were found between the two types of peptic ulcer.

In seven of 11 patients the histamine gastric juice digested proteins other than egg albumen with three maxima below *p*H 5. The maxima occurred at *p*H 1.5 to 2.1, 2.5 to 3.1, and 3.3 to 3.8. The remaining four patients exhibited normal *p*H activity curves with two maxima in the *p*H ranges 1.6 to 2.4 and 3.3 to 4.0.

The gastric juice of three patients digested egg albumen with two maxima, at *p*H 1.5 to 1.6 and 2.6 to 3.1, in contrast to the single maximum for this substrate exhibited by normal subjects.

Whole and fundic mucosal extracts from 12 of 16 patients also digested proteins other than egg albumen below *p*H 5 with three maxima, which occurred at *p*H 1.5 to 2.2, 2.5 to 3.2, and 3.3 to 3.9. The fundic extracts of two patients digested egg albumen with two maxima at *p*H 1.5 to 1.7 and 2.5 to 3.1.

The pyloric mucosal extracts of three of nine patients digested proteins with three maxima at *p*H 1.7 to 1.9, 2.5 to 2.8, and 3.3 to 3.8, but in the other six the normal pyloric pattern of two maxima, at *p*H 1.7 to 2.1 and 2.8 to 3.4, was observed.

The gastric juice of two patients with peptic ulcer and the fundic mucosal extract of a third hydrolysed the specific pepsin substrate benzyl-oxycarbonyl-L-glutamyl-L-tyrosine with a single maximum at *p*H 3.6 to 4.0, resembling that exhibited by normal subjects.

Separation by salt fractionation of individual enzymes responsible for individual *p*H maxima was not achieved.

Proteinase activity was present over the *p*H range 6.4 to 7.4 in three of six extracts containing fundic mucosa and in three of five containing pyloric mucosa. With two extracts a maximum at *p*H 7.2 was observed.

Hypotheses that would account for *p*H activity curves with three maxima are discussed. The available evidence favours the formation and secretion by the mucous membrane of an abnormal enzyme or enzymes.

The differences between normal subjects and patients with peptic ulcer would be explained if peptic ulceration occurred more readily in the small group of normal subjects who digest proteins with three maxima than in the remainder of the population.

I wish to thank Miss Barbara Hunt and Mr. Brian Collett for skilful technical assistance, the surgeons of the Radcliffe Infirmary for access to patients and post-operative material, Dr. P. W. Kent for a gift of benzyloxycarbonyl-L-glutamyl-L-tyrosine, and Mr. J. R. P. O'Brien and Dr. R. B. Fisher for reading and criticizing the D.M. thesis from which the material of this paper is abridged.

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"MYELOMA" SERUM ELECTROPHORETIC PATTERNS IN CONDITIONS OTHER THAN MYELOMATOSIS

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In most patients with myelomatosis, the serum electrophoretic pattern characteristically contains an anomalous component. Occasionally, however, patterns of the "myeloma" type are found in patients in whom the clinical findings and laboratory evidence do not support a diagnosis of myelomatosis.

In this paper, the case histories of 10 such patients are presented and the significance of the findings discussed. Three of these cases have been referred to already (Owen and Rider, 1957).

Methods

Paper Electrophoresis.—Except in Cases 5 and 7 this was carried out as described by Owen and Rider (1957). Sera from Cases 5 and 7 were examined by a method essentially that described by Flynn and de Mayo (1951). Quantitative results were obtained by elution of dye from stained electrophoretic strips. Total protein was determined by the method of Gornall, Bardawill, and David (1949).

Starch Gel Electrophoresis.—This was carried out as described by Smithies (1955) and was used to estimate (visual comparison with normal patterns) the amount of "normal" γ-globulin in sera with abnormal components migrating as γ-globulin.

Ultracentrifugal Analysis.—Sera, diluted 1:4 in 0.2 M sodium chloride (except in Case 10), were examined in a "spinco" model E ultracentrifuge. In Case 10 the serum was diluted 1:2 in 0.15 M sodium chloride. Sedimentation constants were expressed as $S_{20,w}$ values, but were not corrected to zero concentration. For descriptive purposes, the larger (slower) of the two main components of normal serum has been termed "albumin" peak and the smaller (faster) component "globulin" peak.

Precipitin Reactions.—We are indebted to Dr. L. Korngold, Sloan-Kettering Institute, New York, for permission to record his results obtained by a method already described (Korngold and Lipari, 1956).

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Case Histories

Case 1.—C. M., a man aged 73 years, was admitted to hospital in March, 1956, suffering from melaena. He had a duodenal ulcer demonstrated radiologically; he also suffered from pneumoconiosis. There was no clinical enlargement of liver, spleen, or lymph nodes. Sternal marrow was found to be normal. Radiological examination of the skeleton revealed no abnormality apart from changes suggestive of Paget's disease in the right ilium.

Serum paper electrophoresis revealed an abnormal component migrating between β- and γ-globulin (Fig. 1). Ultracentrifugal analysis of serum showed a normal pattern (Fig. 2a). The results of biochemical and haematological findings are summarized in Table I. There was no proteinuria.

During the next two years the patient was well apart from exertional dyspnoea due, presumably, to the pneumoconiosis. Radiological findings remained unchanged. Re-examination of sternal marrow showed a slight increase in plasma cells, but none were atypical.

In May, 1958, the patient developed obstructive jaundice. At this time an aspiration biopsy of a spinous process revealed normal marrow. At operation, the common bile duct was found to be dilated, due, apparently, to a lesion in the head of the pancreas. A palliative cholecyst-jejunostomy was performed, but the patient developed hepatic failure and died. Necropsy revealed a constriction of the common bile duct as a result of fibrosis in the head of the pancreas consequent on the presence of a peptic ulcer in the second part of the duodenum. Microscopic examination of liver, spleen, lymph gland, and bone marrow showed no abnormality apart from mild lymphocytic infiltration of the portal tracts.

Case 2.—A. W., a man aged 42 years, was admitted to hospital in May, 1955, for investigation of attacks of bronchitis, pneumonia, and pleurisy, which had recurred on 14 occasions during the previous 20 years. No cause was found for the recurrent chest infections. There was no enlargement of liver, spleen, or lymph nodes. Bone marrow examination was normal. Radiographs of the skeleton were

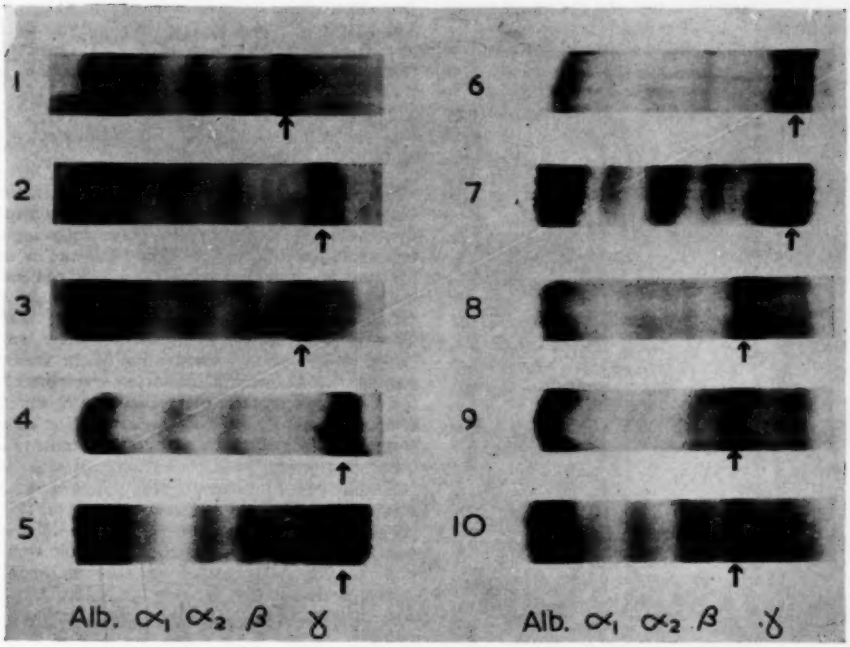

FIG. 1.—Electrophoretic patterns of serum proteins. Arrows indicate the abnormal components.

normal except for an area of translucency in the left ilium, thought to be due to a congenital cyst.

Serum paper electrophoresis revealed the presence of a discrete component migrating as a γ-globulin (Fig. 1). Starch gel electrophoresis and ultracentrifugal analysis were not carried out, but paper electrophoresis showed little "normal" γ-globulin. Biochemical and haematological findings are summarized in Table I.

The patient has continued to have attacks of chest infection but has not developed any further evidence of multiple myelomatosis.

Case 3.—A. C., a man aged 56 years, was admitted to hospital in October, 1957, suffering from pneumonia. He had had three attacks of pneumonia in the previous five months. Six years previously he had fractured his spine in a car accident. There was no clinical enlargement of liver, spleen, or lymph nodes. Bone marrow examination from the sternum revealed 4% plasma cells showing normal morphology. Radiographs of the skeleton were normal except for some osteoporosis of the vertebral bodies and flattening of the sixth thoracic vertebra, thought to be due to his previous back injury.

TABLE I
INITIAL BIOCHEMICAL AND HAEMATOLOGICAL FINDINGS

Case	Serum Proteins (g./100 ml.)						Precipitin† Reaction	Hb (g./100 ml.)	Leucocytes (10^3/c.mm.)					Platelets (10^3/c.mm.)	E.S.R.‡ (mm./1 hr.)
	Total	Albumin	α_1	α_2	β	γ*			Total	N.	L.	M.	Others		
1	5·7	3·1	0·3	0·4	0·4	1·5	MM I	13·0	5·3	4·4	0·5	0·2	0·2	—	15
2	9·2	2·7	0·4	0·5	0·5	5·3	—	10·6	4·8	1·8	2·7	0·2	0·2	—	130
3	6·7	3·2	0·4	0·5	0·7	1·9	MM I	11·6	5·5	3·0	1·6	0·5	0·4	250	50
4	8·8	2·7	0·2	0·3	0·3	5·3	MM II	10·1	6·4	3·8	2·3	0·2	0·1	330	138
5	7·6	4·0	0·4	0·5	0·6	2·0	—	8·4	3·1	2·1	0·6	0·3	0·1	220	26
6	11·0	2·5	0·3	0·6	0·7	7·1	—	3·5	2·5	1·6	0·7	0·2	—	77	152
7	7·3	3·1	0·1	0·5	0·7	2·8	—	9·1	6·6	3·5	2·4	0·5	0·2	400	126
8	7·2	2·3	0·3	0·5	0·4	3·7	Macro-globulin	11·4	6·8	5·8	0·5	0·4	0·1	162	121
9	7·5	3·2	0·5	0·2	0·7	2·9	MM III	6·8	19·0	17·0	1·0	1·0	—	150	150
10	7·8	4·6	0·2	0·4	1·0	1·5	Normal	14·1	—	—	—	—	—	—	30

* Including abnormal component. † See Korngold and Lipari (1956). ‡ Westergren method except in Cases 3 and 5, in which Wintrobe's method was used.

Serum paper electrophoresis showed a discrete component in the γ-globulin position (Fig. 1). The amount of "normal" γ-globulin apparent on starch gel electrophoresis was not significantly reduced. The ultracentrifugal pattern was normal during the initial part of the run (Fig. 2a); later patterns (Fig. 2b) showed a slight increase in the globulin peak.

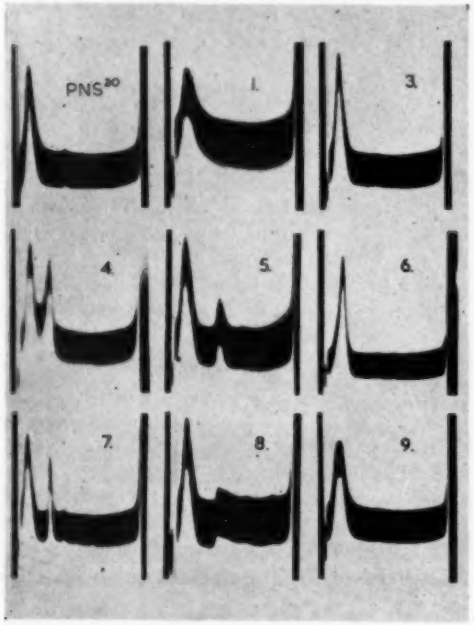

FIG. 2a.—Ultracentrifugal patterns of sera. Patterns are those obtained at 20±2 min. after reaching constant speed (60,000 r.p.m., except in Case 7 where the speed was 50,000 r.p.m.). PNS, pattern of pooled normal serum. Figures indicate case number.

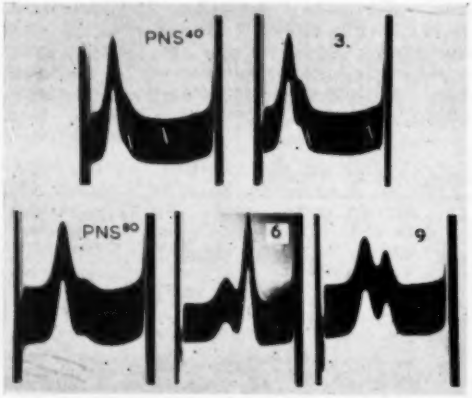

FIG. 2b.—Ultracentrifugal patterns of sera. The patterns were obtained in Case 3, 40 min., and in Cases 6 and 9, 80 min., after reaching constant speed. The patterns of pooled normal serum (PNS) were obtained at corresponding times.

The patient was last reassessed in May, 1958. He was well with no anaemia. His serum electrophoretic pattern was as before.

Case 4.—V. T., a woman aged 58 years, has been known to have pulmonary tuberculosis in a quiescent form since 1953. In 1953 a persistently high blood sedimentation rate was noted and she was investigated for possible myelomatosis. This was not substantiated. She was admitted to hospital in July, 1957, suffering from acute meningococcal meningitis. There was no clinical enlargement of liver, spleen, or lymph nodes. *X*-ray examination of the skeleton was normal. Bone marrow aspiration was normal except for a slight increase in plasma cells (5%) with normal lymphocytes of 12%. Histological section of marrow fragments showed that the plasma cells tended to occur in clumps.

Serum paper electrophoresis showed a discrete component in the γ-globulin position (Fig. 1). Starch gel electrophoresis showed that "normal" γ-globulin was not significantly reduced. The ultracentrifugal pattern (Fig. 2a) revealed an abnormal component with a sedimentation constant of 9.4S. Biochemical and haematological findings are summarized in Table I.

The patient was readmitted to hospital in February, 1958, complaining of backache. *X*-ray examinations disclosed an area of rarefaction with anterior wedging of the twelfth thoracic vertebra and a diffuse, fine mottling in the skull. Marrow aspiration again disclosed only a slight increase in plasma cells, not sufficient to be diagnostic of myelomatosis. Serum proteins were as before. The patient was last reassessed in June, 1958, when she was admitted to hospital suffering from lobar pneumonia. There was now a severe degree of collapse of the seventh, eighth, and twelfth thoracic and the third lumbar vertebrae. A third marrow aspiration again showed a slight increase in plasma cells (4.5%) and lymphocytes (16%), but the findings were not sufficient to be diagnostic of myelomatosis.

Since this paper was submitted, in December, 1958, the patient has suffered a fatal subarachnoid haemorrhage. At necropsy partial collapse of the lower thoracic vertebral bodies was found. The bony trabeculae were thin and spongy and the marrow spaces were filled with soft, dark red tissue.

Histologically numerous groups of closely packed, small cells possessing a single, eccentrically placed and deeply staining nucleus were seen. The great majority of these resembled plasma cells. These groups occurred in isolated clumps separated by normal bone marrow. In the osseous trabeculae there was evidence of active bone resorption and accompanying fibrosis.

The appearances were those of multiple myeloma. Failure to obtain a diagnostic aspirate during life could be explained by the patchy distribution of the myeloma cells.

Case 5.—E. H., a woman aged 70 years, was admitted to hospital in December, 1957, suffering from weakness and loss of weight. She was found to be anaemic; there was moderate enlargement of the liver and spleen and generalized lymphadenopathy. Bone marrow examination from the sternum showed a marked infiltration with lymphocytes. A biopsied lymph node showed the histological picture of lymphosarcoma.

Serum paper electrophoresis revealed an abnormal component with the mobility of γ-globulin (Fig. 1). Biochemical and haematological findings are summarized in Table I. The ultracentrifugal pattern (Fig. 2a) revealed an abnormal component with a sedimentation constant of 15.4S.

The patient was last reassessed in June, 1958. She felt well and the anaemia had improved following therapy with prednisolone. Lymphadenopathy was still present, but the liver and spleen were reduced in size. Ultracentrifugal analysis of serum showed the abnormal component to be still present but reduced in amount.

Case 6.—D. M., a woman aged 60 years, was admitted to hospital in January, 1958. She had suffered from pneumonia three months previously and had not been well since then. Clinical examination revealed pallor, marked enlargement of both liver and spleen, but no lymphadenopathy. Skeletal radiographs showed punched-out osteolytic lesions in the skull but not elsewhere. Two attempts at bone marrow aspiration were unsuccessful (dry tap). A histological preparation from surgical biopsy of iliac crest was reported as follows:

> "The bone marrow is hypercellular due to widespread infiltration with small round cells, mainly lymphocytes, but mixed with these are cells with the appearance of atypical plasma cells. There is plentiful fibroblastic connective tissue. Conclusion: Lymphosarcoma or some closely related condition."

Serum paper electrophoresis revealed an abnormal protein with the electrophoretic mobility of γ-globulin (Fig. 1). Biochemical and haematological findings are summarized in Table I. The ultracentrifugal pattern was normal during the initial part of the run (Fig. 2a); later (Fig. 2b) it was seen that the globulin peak was considerably increased with a marked inversion of the albumin/globulin ratio.

The patient was last reassessed in August, 1958. She has required repeated blood transfusions to maintain an adequate level of haemoglobin in the blood. The clinical findings have not changed.

Case 7.—E. W., a woman aged 75 years, has been described in detail elsewhere (Pitney, O'Sullivan, and Owen, 1958). She suffers from a refractory anaemia of long duration, possibly 15 years. Neither the liver nor spleen are clinically enlarged. One enlarged axillary lymph gland is present. Skeletal radiographs are normal. The bone marrow contains 29% lymphocytes and 2% plasma cells.

An abnormal serum protein with the electrophoretic mobility of γ-globulin (Fig. 1) was discovered in 1955. Starch gel electrophoresis showed a reduction in normal γ-globulin. The ultracentrifugal pattern (Fig. 2a) revealed an abnormal component with a sedimentation constant of 15.3S. Biochemical and haematological findings are summarized in Table I.

This patient is remarkable in that the anaemia has been corrected on two occasions by prednisolone therapy, even though it is not haemolytic in type. After six months of continuous therapy with prednisolone, the concentration of abnormal protein diminished in the serum. Over the past two years she has suffered from episodes of furunculosis, orbital cellulitis, and acute pyelonephritis. The patient was last reassessed in August, 1958. The haemoglobin value of the blood was satisfactory and the patient felt well. She is taking a maintenance dose of prednisolone.

Case 8.—J. W., a man aged 66 years, was admitted to hospital in September, 1957, suffering from lobar pneumonia. He had had a previous attack of pneumonia five years before but had been well in the meantime. He failed to respond to therapy with antibiotics and the pneumonic process spread to involve other lobes. He developed anuria and died in uraemia, eight days after admission to hospital. There was no clinical enlargement of liver, spleen, or lymph nodes. Bone marrow examination (sternum) showed an increase in plasma cells (9%). These were normal morphologically. Fifteen per cent. of the nucleated cells were lymphocytes. Skeletal radiographs were not taken and necropsy was refused.

Serum paper electrophoresis revealed an abnormal component migrating as a γ-globulin. Biochemical and haematological findings are summarized in Table I. Ultracentrifugal analysis of serum revealed the presence of three macroglobulin components (Fig. 2a). The most prominent of these had a sedimentation constant of 16.3S.

Case 9.—S. N., a woman aged 86 years, was admitted to hospital in March, 1957, suffering from auto-immune haemolytic anaemia. Anaemia was severe (Hb = 6.4 g./100 ml.), with a reticulocyte count of 20%; the direct Coombs test on the red cells was positive and free agglutinin was detected in the serum, active in both saline and albumin at 5° C., but in albumin only at 37° C. Results of the γ-globulin Coombs neutralization test were of the cold agglutinin type.

Serum paper electrophoresis revealed an abnormal protein migrating between β- and γ-globulin (Fig. 1). Biochemical and haematological findings are summarized in Table I. There was no proteinuria. Ultracentrifugal analysis revealed a reduced albumin peak with an increased globulin peak.

The patient did not improve with transfusions or steroid therapy and died five days after admission to hospital. Necropsy failed to reveal a cause for either the auto-immune haemolytic anaemia or the abnormal serum protein. There was neither macroscopic nor microscopic evidence of myelomatosis, the bone marrow showing hyperplasia only when examined microscopically.

Case 10.—A. W., a woman aged 66, was admitted to hospital in August, 1956, with a swelling on the chest wall attached to a rib. Radiological examination revealed destructive lesions in several ribs. Liver, spleen, and lymph nodes were not enlarged and biopsy of sternal marrow showed no abnormality.

Electrophoresis of serum demonstrated an abnormal component migrating between β- and γ-globulin (Fig. 1). Other biochemical findings are listed in Table I. An ultracentrifugal analysis showed a distinct macroglobulin component, but this was considered to be too small to be of pathological significance.

The histology of a biopsy from the presenting swelling was strongly suggestive of it being a secondary deposit from a thyroid carcinoma. Biopsy of the thyroid gland confirmed malignancy, and on this account an almost complete thyroidectomy was performed. Examination of the operation specimen confirmed the diagnosis. The abnormal serum component was present undiminished one month after the operation. Unfortunately, it has not been possible to follow up this case.

Discussion

The diversity of findings in the above cases at once suggests that the patients did not all suffer from the same disease. In each case, a diagnosis of myelomatosis was considered initially because of an abnormal discrete component in the serum electrophoretic pattern. In some patients, e.g., Cases 2, 3, and 4, this diagnosis has yet to be excluded. It is well known that myelomatosis may run a protracted course and characteristic skeletal changes may not appear until late, if at all. Thus a patient (T. R.) mentioned briefly in a previous report (Owen and Rider, 1957) did not develop conclusive signs of myelomatosis until two years after an abnormal component was first detected in the serum.

In Case 1, necropsy failed to provide any explanation for the electrophoretic anomaly which appears to have been an incidental finding.

Cases 2 and 3 are both middle-aged men who suffer from repeated lung infections associated with mild anaemia. In Case 2, a lesion in the left ilium remains suspect as a myeloma deposit, although the *x*-ray appearances have not altered over a period of three years. In Case 3, the bone marrow contained 4% plasma cells which, however, were normal morphologically. In neither case, however, is myelomatosis ruled out.

It is also possible that Case 4 suffers from myelomatosis although the history and findings are unusual. The abnormal serum component (sedimentation constant=9.5S) has a molecular weight intermediate between that of the usual myeloma protein (sedimentation constant=6–7S, Putnam, 1957) and what are termed "macroglobulins" (sedimentation constant=15–25S). However, relatively high molecular weight proteins have been found occasionally in association with myelomatosis (Waldenström, 1952; Putnam and Udin, 1953; Wanner and Seibenmann, 1957).

The more usual form of Waldenström's macroglobulinaemia (Waldenström, 1944) is well exemplified by Case 5. Case 6 is, in some ways, similar; there is enlargement of liver and spleen, with lymphocytic infiltration of the bone marrow. However, there is no enlargement of lymph nodes and the bone marrow shows fibrosis as well as lymphocytic infiltration; further, the abnormal serum protein is not a macroglobulin. Several authors (e.g., Azar, Hill, and Osserman, 1957; Mackay, Taft, and Woods, 1957) have reported the association of a myeloma-like serum electrophoretic pattern with malignant lymphoma.

In Cases 7 and 8, the abnormal serum proteins were macroglobulins. In Case 7, the only abnormal finding, apart from anaemia, was an enlarged axillary gland which has remained stationary in size for two years. Like Cases 2, 3, and 4, this patient has shown a susceptibility to infection. Case 8 was well before his terminal acute illness and the demonstration of macroglobulin seems to have been an incidental finding. It was particularly unfortunate that necropsy was refused in this case.

The varied clinical findings in Cases 5, 7, and 8 confirm previous conclusions (Martin and Close, 1957) that macroglobulinaemia is probably not associated with any one disease.

No underlying cause was found for the haemolytic anaemia in Case 9. It has been reported, however, that certain patients with idiopathic auto-immune haemolytic anaemia of the cold agglutinin type have an abnormal electrophoretic component migrating as a fast γ-globulin (Christenson and Dacie, 1957; Fudenberg, Barry, and Dameshek, 1958). Case 9 probably falls into this group. In such cases it appears that the abnormal component constitutes the agglutinating substance (Christenson, Dacie,

and Croucher, 1957). An association of abnormal serum components with haemolytic anaemia has also been noted in patients with lymphoma (Christenson and Dacie, 1957) and chronic lymphatic leukaemia (Craig, Waterhouse, and Young, 1952; Buffa and Rappaport, 1957).

In Case 10, the abnormal protein was associated with a carcinoma of the thyroid. Kanzow (1954) and Schaub (1953) have each described single cases of carcinoma of the uterus associated with abnormal serum components (in both cases macroglobulins). However, there was evidence in both cases of an associated lymphoma.

Cases 2 and 3, and to a lesser extent Cases 4 and 7, showed a susceptibility to infection. Whilst it is tempting to ascribe this to a deficiency of normal γ-globulin, it must be admitted that in none of these was the reduction in γ-globulin severe. Hypo-γ-globulinaemia has frequently been noted in persons with myelomatosis (Adams, Alling, and Lawrence, 1949; Rundles, Cooper, and Willett, 1951; Snapper, Turner, and Moscovitz, 1953; Waldenström, 1954; Zinneman and Hall, 1954; Lawson, Stuart, Paull, Phillips, and Phillips, 1955; Porges, 1956; Firkin and Blackburn, 1958), and it has been suggested that the formation of abnormal serum protein competes with the formation of normal γ-globulin. However, it should be noted that hypo-γ-globulinaemia has also been reported in reticuloendothelial disease in the absence of abnormal serum components, notably in chronic lymphatic leukaemia (Jim and Reinhard, 1956; Wall, 1956), in malignant lymphoma (Arends, Coonrad, and Rundles, 1954), and in diffuse reticuloendothelial hyperplasia (Citron, 1957).

Korngold and Lipari (1956) have recorded that myeloma sera can be divided into three groups according to the ability of the myeloma protein to react with antiserum prepared against normal γ-globulin. Sera from four of the six cases tested (Table I) gave reactions similar to those given by myeloma sera. In two of these patients (Cases 1 and 9), however, the results of necropsy examination did not support a diagnosis of myelomatosis, which rather suggests that the precipitin reactions obtained by Korngold and Lipari (1956) are not specific to myelomatosis.

It is inappropriate to consider here at length the pathogenesis of the abnormal serum components. However, there are two points which deserve comment. In several of the cases of the present series (Nos. 1, 2, 3, and 9) there was no evidence of neoplastic disease either of the reticuloendothelial system or of other tissue. The occurrence of abnormal serum proteins in the absence of neoplasia is apparently rare. Apart from cases of cold-agglutinin haemolytic anaemia we have been able to find only a few reports of such cases. These include several well-documented cases of essential cryoglobulinaemia (Hutchinson and Howell, 1953; Barnett, Curtain, Hayes, Holden, Pye, and O'Donnell, 1956; Volpé, Bruce-Robertson, Fletcher, and Charles, 1956), and a few other cases more briefly reported, with diagnoses of hepatitis (Wieme, 1953) and unexplained anaemia (Flynn, 1954; Sonnet, Louis, and Heremans, 1955) respectively. Of particular interest is the report (Laurell, Laurell, and Waldenström, 1957) of this anomaly in a blood donor who showed no other physical or laboratory signs of disease.

Secondly, the finding that abnormal electrophoretic components behave, in some instances, as auto-immune agglutinating antibodies is possibly important in any consideration of their pathogenesis. It is possibly also relevant that very high titres of auto-immune complement-fixing antibodies have been found in some sera containing large amounts of macroglobulin (Mackay and Gajdusek, 1958). On the other hand, not every case of auto-immune haemolytic disease has such anomalous protein components. Further, in Hashimoto's disease, which also is characterized by the presence in serum of auto-immune antibodies (Witebsky, Rose, and Shulmann, 1958; Roitt, Doniach, Campbell, and Hudson, 1956) the electrophoretic pattern does not reveal anomalous components although the γ-globulin component is often increased (Luxton and Cooke, 1956; Doniach and Hudson, 1957). This, however, may simply reflect the inadequate resolving powers of prevailing electrophoretic methods.

Summary

Ten patients are described in whom the serum electrophoretic pattern was of the myeloma type but in whom a diagnosis of myelomatosis was not established. In four patients the abnormal protein was a macroglobulin.

In five patients there was evidence of reticuloendothelial disease whilst one patient suffered from a carcinoma of the thyroid gland. In the other patients there was no evidence of neoplasia.

Four patients showed an increased susceptibility to infection which was possibly related to hypo-γ-globulinaemia.

It is concluded that whilst discrete abnormal electrophoretic components in serum are most often associated with myelomatosis or lymphoma, they are occasionally present in a variety of other conditions.

We should like to thank the physicians who have allowed us access to patients in their care and the many others who have helped in this investigation. The technical assistance of Mr. G. S. Wilson, Commonwealth Serum Laboratories, in performing ultracentrifugal measurements is gratefully acknowledged. During part of the investigation, financial support was provided for one of us (J. A. O.) by the National Health and Medical Research Council of Australia, to which we are indebted.

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BLANKET LAUNDERING AND STERILIZATION

REPORT OF A TRIAL AT THE CITY GENERAL HOSPITAL, SHEFFIELD

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Trials have recently been carried out at the City General Hospital, Sheffield, with various types of blankets as follows:

(1) All wool blankets to B.S. 1681: 1951, nominal size 76 in. × 96 in., weight 5 lb. 2 oz., cost 53s. 10d. each.

(2) All wool blankets to B.S. 1681: 1951, nominal size 76 in. × 96 in., weight 5 lb. 2 oz., cost 53s. 10d. each, treated for shrinkage resistance at an approximate cost of 7s. 1½d. per blanket.

(3) Cellular cotton blankets (loom state), nominal size 76 in. × 96 in., weight 3 lb. 6 oz., cost 29s. 11d. each.

(4) Turkish towelling blankets (bleached), nominal size 80 in. × 100 in. made from two pieces 100 in. × 40 in. joined up the centre, weight 5 lb. 4 oz. per blanket, cost 66s. 6d. each.

Preparation

All blankets were laundered before being put into use in the wards. This was particularly necessary in the case of the cellular cotton blankets as these were supplied in the loom state.

A small patch of white material, 6 in. by 3 in., was stitched on one corner of each blanket and a mark was placed thereon after each laundering.

Distribution

All blankets were put into use on January 7, 1958.

Ward 2A (16 beds).—Three cellular cotton blankets per bed were allocated at a total cost of £4 9s. 9d. per bed.

Ward 2B (21 beds).—Two wool blankets and one treated wool blanket per bed were allocated at a total cost of £8 8s. 7½d. per bed.

Ward 2C (21 beds).—Two cellular cotton blankets and one Turkish towelling blanket were allocated per bed at a total cost of £6 6s. 4d. per bed.

Equipment and Materials Used

Washing Machine.—A 100-lb. dry weight open-end washer fitted with interrupter gear by James Armstrong & Co.

Hydro Extractor.—A 36-in. J.B. by Thomas Broadbent, Huddersfield. Speed 1,440 revolutions per minute.

Drying Machine.—A Lister air speed drier with a continuous dry room temperature 200–212° F. where the blankets are hung by clips on a continuous belt taking 15 minutes to pass through (Lister Bros., London).

Syndet 54.—A powdered non-ionic detergent combined with a mildly acid builder, used at the rate of 2 lb. per 70 lb. load, manufactured by Laporte Chemicals Ltd., Luton.

Steravol.—A quaternary ammonium compound, cetyl trimethyl ammonium bromide, using 2 pints per 70 lb. load, supplied by Laporte Chemicals Ltd.

Escolite N.—An alkaline blended product of high active alkalinity; at 70° F. a 0.1% solution has a *p*H value of 11.6 (Laporte Chemicals Ltd.).

Comprox TL.—An anionic detergent (British Petroleum Products Ltd.).

Pure Soap.—Chips or powder at a high titre for use at high temperatures.

Cotton Wash Solution.—For cleaning the cotton blankets a solution was made up of 800 gallons water, 36 lb. "escolite," 10½ lb. "comprox TL," and 7 lb. pure soap. This was pumped into the machine at 2 gallons per minute.

Laundering

Woollen blankets, untreated and treated, were given a 10-minute wash at about 100° F. in "syndet 54" and a five-minute cold rinse, both on interrupted gear. The blankets were next treated for five minutes with "steravol." The blankets were put into a clean canvas trolley and transferred to a hydro extractor where they were rough-dried for eight minutes. They were then replaced in the clean canvas trolley and taken to the continuous hot air drier and dried at 200 to 212° F. (air temperature) for 15 minutes.

Cellular cotton blankets and Turkish towelling blankets were given a 10-minute wash at 160° to 170° F. and at 180° to 200° F. respectively using a solution of "escolite," "comprox TL," and pure soap (chips or powder). This was followed by a continuous cold rinse for six minutes. The blankets

TABLE I
LAUNDERING PROCESSES

	Temperature (°F.)	Time (min.)
*Wool**		
1. Wash, "syndet 54"	100	10
2. Rinse, water	Cold	5
3. Sterilized, "steravol"	,,	5
4. Hydro extraction		8
5. Dried on continuous belt (air temperature)	200–212	15
Cotton		
1. Wash ("escolite," "comprox TL," and soap):		
(a) Cotton cellular	160–170	10
(b) Turkish towelling	180–200	
2. Rinse	Cold	6
3. Hydro extraction		10
4. Dried on continuous belt (air temperature)	200–212	15

* This process is slightly modified to that originally recommended by Laporte Chemicals Ltd.

were then transferred in a clean canvas trolley and treated in a hydro extractor for 10 minutes and then transferred to the air drier where they were dried like the woollen blankets for 15 minutes at 200 to 212° F. (air temperature). The processing is summarized in Table I.

Findings after Ward Use and Laundering

Untreated wool blankets received normal usage reports. After 15 washings the blankets showed some felting and slight dullness of colour.

Treated wool blankets also received normal usage reports. After 15 washings the blankets were of good appearance and in moderately good condition.

Cellular cotton blankets caused "fluff" originally, but this gradually ceased. In a few blankets some loose threads were seen and several end seams became unstitched at the end of the first wash. The patients were glad to have the light blankets and the nursing staff found them better in bed-making than woollen blankets. After 10 launderings done according to the prescribed procedure the blankets were rather hard to the touch. After five more washings, the blankets, having been dried as a comparison in a tumbler dryer each time, were much softer to the touch.

Turkish towelling blankets were much heavier than woollen and not so warm. They were more difficult to handle owing to their extra length and

TABLE II
MEASUREMENTS AND SHRINKAGE CALCULATIONS

	Size			Shrinkage from Original Size			Weight lb. oz.	Remarks
	Length (in.)	Width (in.)	Area (sq. in.)	Length (%)	Width (%)	Area (%)		
Untreated Wool Blankets								
Original size	96·70	78·60	7,600	—	—	—	5 2	Average of 10 blankets
After 1 wash	95·40	76·10	7,260	1·34	3·18	4·47	4 15	,, ,, 5 ,,
,, 5 washes	94·45	74·08	6,996	2·32	5·75	7·95	4 13	,, ,, 12 ,,
,, 10 ,,	88·33	69·00	6,095	8·66	12·21	19·80	4 13	,, ,, 5 ,,
,, 15 ,,	87·20	68·50	5,973	9·82	12·85	21·41	4 12	,, ,, 5 ,,
Treated Wool Blankets								
Original size	96·70	78·60	7,600	—	—	—	5 2	,, ,, 10 blankets
After treatment	95·22	75·75	7,213	1·53	3·63	5·09	4 10½	,, ,, 5 ,,
,, 5 washes	94·33	73·66	6,948	2·45	6·28	8·58	4 4	,, ,, 9 ,,
,, 10 ,,	93·45	72·87	6,810	3·36	7·29	10·39	4 3½	,, ,, 4 ,,
,, 15 ,,	92·83	72·75	6,753	4·00	7·44	11·14	4 3½	,, ,, 4 ,,
Cellular Cotton Blankets								
Original size	96·00	76·00	7,296	—	—	—	3 6	,, ,, 9 blankets
After 1 wash	92·28	74·14	6,842	3·88	2·45	6·22	3 4½	,, ,, 5 ,,
,, 5 washes	93·80	73·60	6,903	2·29	3·16	5·39	3 4	,, ,, 5 ,,
,, 10 ,,	88·50	62·66	5,545	7·81	17·55	24·00	3 4	,, ,, 5 ,,
(a)	89·56	68·94	6,174	6·70	9·29	15·38	—	(a) After stretching the same blankets in a dry condition
,, 15 ,,	84·60	61·20	5,178	11·87	19·47	29·03	3 4	Average of 5 blankets
(b)	83·50	67·00	5,595	13·02	11·84	23·31	—	(b) After stretching the same blankets in a dry condition
(c)	90·33	73·00	6,594	5·91	3·95	9·62	—	(c) After stretching the smallest blanket (82 in. × 66½ in.) in a wet condition Measurements taken after the blanket was dried at room temperature
Turkish Towelling Blankets								
Original size	100·00	81·00	8,100	—	—	—	5 4	Average of 10 blankets
After 1 wash	100·28	80·00	8,022	—	—	0·96	5 3	,, ,, 7 ,,
,, 5 washes	100·14	79·71	7,982	—	—	1·45	5 3	,, ,, 7 ,,
,, 10 ,,	99·80	77·00	7,685	0·20	4·94	5·12	5 5	,, ,, 5 ,,
,, 15 ,,	100·50	76·50	7,688	—	5·55	5·09	5 5	,, ,, 5 ,,

width. After 15 washings the blankets were of good appearance and normal texture.

The measurements and weights taken on woollen blankets and cotton blankets at various stages and also the shrinkage calculations are shown in Table II.

Both patients and nurses prefer the cellular cotton blankets to the Turkish towelling. They are cheap, easily laundered, and are giving satisfactory service after 11 months' wear. Moreover they combine the qualities of lightness and warmth.

The cellular cotton blankets shrank excessively. About 20 to 30% of this shrinkage could be recovered by stretching in the dry state, and a further 40% approximately by stretching in the wet state, but leaving a final shrinkage of 9% of the original size. These blankets also stretch somewhat in the normal process of bed-making. The laundering of cellular cotton blankets, if adopted, would have to be carefully controlled in the laundry.

The untreated woollen blankets were only fairly satisfactory as they shrank considerably with some felting and loss of colour.

The treated woollen blankets were better in all respects than the original wool blankets.

Bacteriological Examinations

Blankets were sampled by the "sweep plate" technique as described by Blowers and Wallace (1955). The area sampled was the same for all blankets. The blanket was laid on a laundry trolley which was covered with a clean sheet. The same method of sweeping was made each time, first in the shape of a St. George's cross (+) and then of a St. Andrew's cross (×). The size of the side of the trolley was 26 in. × 34 in. and the length of the sweep was approximately 10 ft. Petri dishes, 4 in. diameter, were used containing nutrient agar with ½% "lubrol" (I.C.I.) and ¼% sodium stearate in order to inactivate the effect of any residual quaternary ammonium compound which might inhibit bacterial growth. The plates were counted after 24 hours' aerobic incubation. It was noticed that 48 hours' incubation produced larger colonies but did not increase the colony count appreciably, so that 24 hours was taken as the standard incubation time.

Preliminary sweep plate cultures before laundering of all types of blankets under test gave counts of 300 to >500, usually uncountable.

Before laundering many pathogens could be isolated, especially *Staph. aureus* (coagulase positive), *Bact. coli*, and *Proteus*. After laundering no pathogens were isolated.

A preliminary test of the detergent used in the laundry, "syndet 54," showed that most of the blankets were nearly sterile and some were sterile. The bacteriological results of the trial are shown in Table III.

TABLE III
BACTERIOLOGICAL RESULTS OF THE TRIAL

Blanket	Laundry Process	Wash Temperature (°F.)	No. Tested	Average Sweep Count
Wool ..	Syndet 54 alone	100	50	16
" ..	" " " + "steravol"	100	80	5
Cotton, cellular	Escolite, "comprox TL," and pure soap	160–170	50	3
Turkish towelling	Escolite, "comprox TL," and pure soap	180–200	50	4

Syndet 54 when used alone appears to wash out the dirt and remove practically all bacteria, leaving an article nearly sterile. The results obtained show that this process reduces most of the potential danger of contaminated blankets being returned to the ward.

Syndet 54 plus "steravol," on the other hand, produces an article as nearly sterile as could be obtained under the conditions of sampling. The sterilizing solution probably killed any pathogens not removed by the detergent alone. Although quaternary ammonium compounds are not very effective against *Ps. pyocyanea* this organism was not isolated, in spite of the fact that it was responsible for a ward infection during part of the time the trial was in progress.

The laundry process of the two types of cotton blankets at the relatively high temperatures of 160 to 200° F. did produce an article as nearly sterile as could be obtained under the conditions of sampling. As the dirt and bacteria were thoroughly removed in the wash the high temperature apparently killed any remaining vegetative bacteria.

Discussion

Our results are in keeping with those described by Blowers and Wallace (1955), by Blowers, Potter, and Wallace (1957), and by Frisby (1957). The 20% shrinkage of the untreated and 11% of the treated preshrunk woollen blankets was less than that reported by Blowers *et al.* (1957), possibly because an acid washing detergent was used as recommended by the laundry industry. This detergent had been in use in the hospital for some time so that, when the investigation was begun, the blankets returning from the laundry were exceptionally clean and relatively free from

bacteria, giving sweep plate counts of 10 to 20. There had also been no complaints of serious shrinkage; this was borne out by the careful measurements made in the trial.

Obviously if a hospital wishes to continue purchasing new woollen blankets they should be preshrunk. As most of the blankets in hospitals are woollen it is obvious that they must be used until worn out. To prevent excessive shrinkage and to produce a blanket as near as possible to sterility, the method used in this trial is recommended.

Cotton cellular blankets are, however, easier to sterilize by using temperatures near to that of boiling. The shrinkage in this trial appeared excessively high, possibly because the blankets were not deliberately pulled at the time of measurement. However, if they are stretched intentionally much of the shrinkage can be overcome, although there was 9% loss in our experience.

One disadvantage of the loom state type was the excessive fluff, even after one or two washings, which could disseminate bacteria through a ward. Possibly better quality blankets would prevent this. Calnan (1959) reports most favourably on a new type of cotton cellular blanket—a warp-knitted mesh fabric sewn between two outer coverings of a rayon cotton sheet.

The Turkish towelling blankets can also be sterilized by high temperatures and they showed the least shrinkage of the three. However, they appeared to be heavier and more clumsy to handle in the wards and were not popular with those who had to use them, the patients and the nurses.

Since this investigation was started other types of blanket have been on trial in other hospitals. Schwabacher, Salsburg, and Fincham (1958) have shown that woollen blankets can be disinfected by quaternary ammonium compounds with a resultant reduction in the bacterial contamination of the air. They also showed that "terylene," and more so cotton, blankets reduced the air contamination and also the cross-infection, because there was much less fluff. Pressley (1958), who sampled ward dust at bed height, found that 96% of the fibres were from cotton material and not from woollen, but that of the dust swept up on floors the woollen content was nearly 40%. It is clear, therefore, that fluff from wool or cotton blankets is a serious cause of air contamination. Thomas, Liddell, and Carmichael (1958) reported their investigations into the laundry process of a hospital, but as no disinfection by a quaternary compound or by heat was used the blankets after laundering were still contaminated by *Staph. aureus*.

It is felt that more investigation must be made into the temperatures used in washing and in drying as the texture of the blankets made of any material may be affected by too high or even too low a temperature, possibly resulting in felting and shrinkage.

Stark (1959) reports that even woollen blankets may be boiled without resulting felting or shrinkage if the conditions are carefully controlled. He did, however, note some discoloration.

Now that there are satisfactory ways of washing and sterilizing blankets, all blankets should automatically be sent to the laundry whenever a patient goes home or dies and also fairly frequently, possibly every two to three weeks, from patients remaining in hospital for some time, especially when they are suffering from some infective illness whether surgical or medical.

General Conclusions and Recommendations

Cotton cellular blankets proved the most satisfactory. They are cheap, warm, light, and liked by patients and nursing staff. They are easily sterilized by laundering at temperatures high enough to kill bacteria in the vegetative form. To prevent shrinkage it is advisable to stretch them at the time of laundering, preferably while still wet. It is recommended that cotton cellular blankets be purchased in the future.

Woollen blankets are also satisfactory and can be laundered at 100° F. and "sterilized" in a cold rinse by "steravol," a quaternary ammonium compound, most vegetative bacteria being killed. They do, however, to some extent shrink and felt, but if the laundering is carefully controlled and an acid detergent, such as "syndet 54," is used, this may be reduced to a minimum. This process is recommended for all woollen blankets used in hospitals until they require to be replaced.

Our thanks are due to the laundry manager, Mr. E. Jones, and his staff, and to the matron, sisters, and nursing staff who made this investigation possible, and to the representatives of Laporte Chemicals Ltd. for helpful advice.

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FAMILIAL CARDIOMEGALY

BY

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In 1949 William Evans gave the name of familial cardiomegaly to what he believed to be a "distinct syndrome having a definite clinical, cardiographic, and pathological pattern." The essential features were the familial incidence of cardiomegaly without obvious cause and a marked tendency to arrhythmia and heart block with associated palpitation, giddiness, and syncope. Death may be sudden or rapid due to the development of left ventricular failure. In the electrocardiogram the QRS complexes are often exceptionally wide and the T waves inverted. Conspicuous myocardial fibrosis and hypertrophy of the remaining muscle fibres are the striking pathological findings. Both the degree of conduction defect and the prognosis seem to depend on the extent of myocardial fibrosis and cardiac enlargement.

Evans referred to two previous accounts of obscure cardiomegaly occurring in members of the same family published in the case records of the Massachusetts General Hospital in 1942 and by Addarii (1943) and Addarii, Martini, Mahaim, and Winston (1946). Subsequently cases have been published by Davies (1952), Parsons (1952), De Matteis and Ozzano (1954), Campbell and Turner-Warwick (1956), Gaunt and Lecutier (1956), and Paulley, Jones, Green, and Kane (1956). Among these, however, are included cases of cardiomegaly with no supporting family history and others where there is good reason to believe that the cardiac hypertrophy was secondary to acquired valvular disease, congenital cardiac malformations, or toxoplasmosis. In our view the diagnosis of familial cardiomegaly should only be made when other causes of cardiac enlargement have been excluded and when there is a clear supporting family history. Moreover, in the present state of knowledge, it seems wise only to include those cases where the diagnosis has been established by necropsy study in at least one affected member of the family. The reports of seven affected families (15 cases) comply with these criteria and include eight necropsy studies. Only these cases are included in Table I and the subsequent discussion.

The purpose of this paper is to present a further example of a family with cardiomegaly, the three affected members of which died suddenly. Post-mortem studies were made in all three cases although in one details of the necropsy findings are no longer available.

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Case Histories

Case 1.—G. D., a man aged 30 years at death, came home from work one evening in April, 1956, in his usual good health, and while playing with his children in the garden suddenly collapsed and died. There was no history of rheumatic fever or other serious illness in the past. Eight months before his death he sustained a slight injury to the right groin with haematoma formation in the spermatic cord. His wife thought that he was slow to pick up after that accident, but he eventually recovered, and his mother, who was with him on the day before he died, remarked that she thought he was in excellent health. At no time was he heard to complain of dyspnoea, chest pain, faintness, or palpitation.

Necropsy performed by one of us (A. G. R.) on the instructions of the coroner 21 hours after death revealed the following abnormalities.

The body was that of a well-built young male with a previous appendicectomy scar; significant macroscopic findings were confined to the thoracic cavity. The heart was markedly enlarged (weight 500 g.), the hypertrophy mostly affecting the left ventricle, which in parts was nearly 2 cm. thick and appeared at first sight to be of the usual hypertensive type. On closer inspection it was seen that the outer part of the myocardium consisted of pale fibrous tissue almost giving the impression of an outer shell surrounding the inner layer (Fig. 1). Strands of fibrous tissue could be seen penetrating the deeper aspects of the myocardium, but the predominantly peripheral distribution was striking. The compact outer rim was well demarcated from the rest of the myocardium except in the region of the interventricular septum where its discrete pattern became replaced by diffuse fibrotic mottling. The trabeculae carnae, papillary muscles, and chordae tendineae showed no evidence of fibrosis. The right ventricle and both auricles

TABLE I
MAIN CLINICAL FEATURES

No.	Author	Author's Case No.	Age ( ) = Age at Death	Sex	Family Relationship	Main Symptoms	Arrhythmias	Heart Block	Length of History from Onset of Symptoms	Necropsy Findings
1	Case records of M.G. Hosp. (1942)	1	(21)	M	Brother with congestive failure. Sister with cardiomegaly A–V block and congestive failure	Dyspnoea, fatigue, congestive failure		Complete A–V block	6 months	Yes
2	Addarrii *et al.* (1943)	1	(?)	M	Brother of Case 3	Dyspnoea, syncope	Auricular fibrillation	Complete A–V block, branch bundle block	?	,,
3	Addarii *et al.* (1946)	1	(21)	M	Brother of Case 2. Mother died C.C.F. and syncope	Dyspnoea, fatigue, syncope, congestive failure	Auricular fibrillation, paroxysmal tachycardia	Complete A–V block, branch bundle block	15 years	,,
4	Evans (1949)	1	(18)	M	Son of Case 6. Brother of Case 5	Giddiness, paroxysmal tachycardia	Paroxysmal tachycardia, extrasystoles	Branch bundle block	2 months	,,
5	,, ,,	2	20	M	Son of Case 6. Brother of Case 4	Giddiness	Auricular fibrillation, paroxysmal auricular tachycardia	,, ,,	6 ,,	—
6	,, ,,	3	43	F	Mother of 4 and 5. Three of her siblings believed affected	Syncope	Auricular fibrillation, extrasystoles	—	12 years	—
7	,, ,,	8	(16)	M	Son of Case 8	,,	Sinus bradycardia		4½ ,,	Yes
8	,, ,,	App.	42	F	Mother of Case 7	Paroxysmal, tachycardia with occasional syncope	Paroxysmal tachycardia	Prolonged P–R interval, branch bundle block	2 months	—
9	Gaunt and Lecutier (1956)	1	(51)	M	Brother of Case 11	Dyspnoea, syncope	Auricular fibrillation	Complete A–V block	20 ,,	Yes
10	,, ,,	2	23	F	Daughter of Case 11	Arthralgia, slight dyspnoea	—	—	3 ,,	—
11	,, ,,	3	(53)	F	Mother of Case 10. Sister of Case 9	Syncope, dyspnoea	—	Complete A–V block, branch bundle block	4 years +	—
12	Campbell and Turner-Warwick (1956)	1	(23)	M	Son of Case 13	Syncope, palpitation, dyspnoea, severe chest pain	Paroxysmal tachycardia, ventricular extrasystoles	Prolonged P–R, branch bundle block	3 years	Yes
13	,, ,,	2	(29)	F	Mother of Case 12. Eight of her 12 siblings died of heart disease under 40 years	Dyspnoea, congestive failure, syncope	—	Branch bundle block	1 year	—
14	,, ,,	3	(36)	M	Son of Case 15	Syncope	Sinus and nodal bradycardia, nodal or ventricular extrasystoles	Varying degrees of A–V block, branch bundle block	6 months	Yes
15	,, ,,	4	66	F	Mother of Case 14. Her sister, mother, three aunts, and grandmother died of heart disease	Faintness, fatigue, dyspnoea	Paroxysmal auricular flutter, sinus bradycardia	Branch bundle block	40 years	—

appeared unaffected, and the pericardium and endocardium were also macroscopically normal. The aorta merely showed slight atheroma of its first part, and there was no evidence of coarctation. The lungs were engorged and the trachea and bronchi contained a large amount of mucus; all the other organs, including the brain and spinal cord, appeared normal to the naked eye.

Microscopic examination of the left ventricle showed marked hypertrophy of individual muscle fibres associated with gross fibrosis of the myocardium distributed peripherally and corresponding to the area seen with the naked eye. The fibrous strands tended to separate the muscle fibres into bundles (Fig. 2) and many of these isolated lobules of muscle showed degenerative changes similar to those seen in early cell death following ischaemia (Fig. 3); slight infiltration with lymphocytes was seen in occasional areas of fibrosis (Fig. 4). The right ventricle showed a similar pattern of interstitial fibrosis, although of lesser extent, and its distribution was more irregular and was not confined to the

FIG. 1

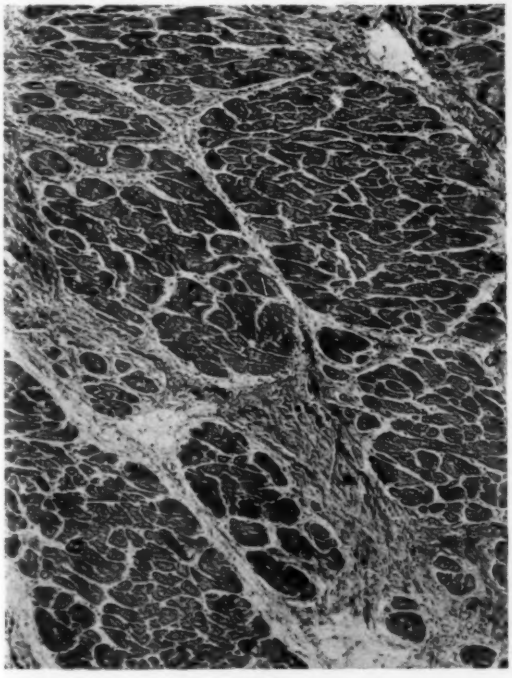


FIG. 2

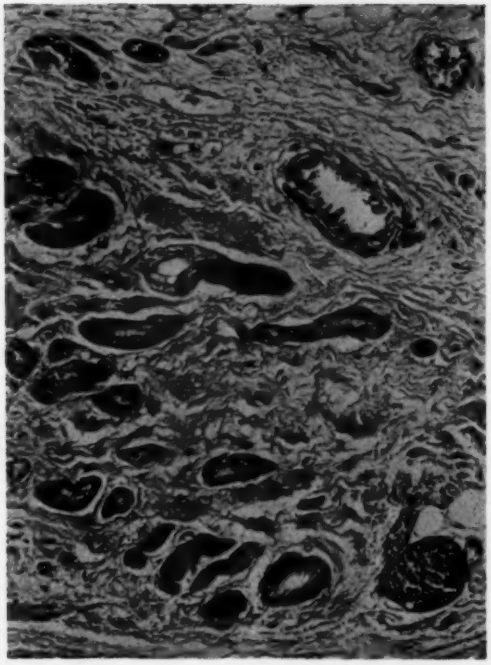


FIG. 3

FIG. 1.—Cross section of left ventricular wall showing peripheral fibrotic mottling in Case 1.

FIG. 2.—Trabeculated fibrosis of the left ventricle in Case 1. Haematoxylin and eosin, × 60.

FIG. 3.—Degenerative change in muscle fibres surrounded by fibrous tissue in Case 1. Haematoxylin and eosin, × 150.

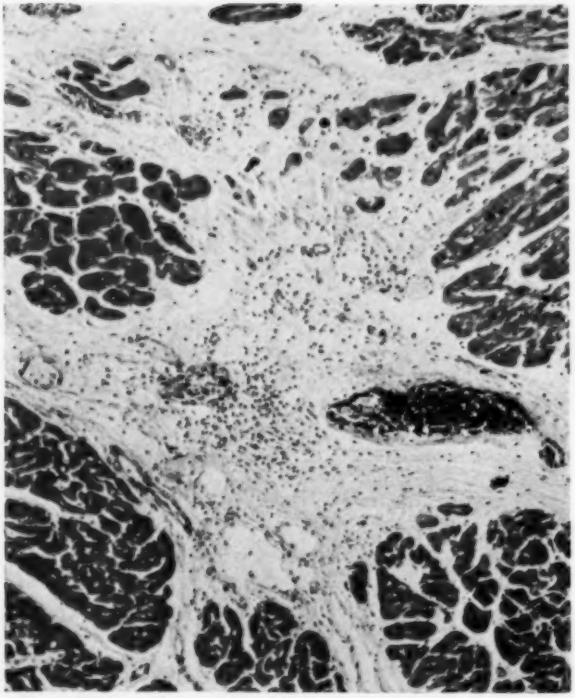


FIG. 4.—Slight lymphocytic infiltration in fibrotic areas in Case 1. Haematoxylin and eosin, × 80.

peripheral areas; the left auricle was normal, but slight fibrotic changes were present in the wall of the right chamber. There was no histological evidence of amyloidosis and stains for amyloid were negative. The endocardium was normal and the coronary arteries and arterioles showed no evidence of disease. Histological examination of the thyroid, testes, kidneys, thymus, pancreas, liver, adrenal glands, pituitary, spleen, brain, spinal cord, and voluntary muscle revealed no significant abnormality. The lungs showed basal venous engorgement and the aorta some soft atheroma.

Case 2.—K. D., the sister of Case 1, died suddenly at the age of 18 years on December 26, 1946. There was no history of previous ill-health. The day before her death was spent working, as usual, as a telephonist at the Post Office. Next morning she felt off-colour, became pale and faint, and complained that her legs were numb. She developed increasing shortness of breath with frothy sputum, white at first but later tinged with pink. She died shortly before midnight.

Her death was the subject of a coroner's inquiry, and the necropsy report gave as the main findings cardiac enlargement and pulmonary oedema. The heart was said to show "a little fibrosis of the papillary tips," but unfortunately the myocardium was not examined histologically. The coronary arteries were recorded as being normal. Various organs were examined for a wide variety of chemical poisons with negative results, death being eventually attributed to "acute pulmonary oedema precipitated by streptococcal pneumonia." From the description of the lungs and heart it seems clear that in fact death was due to left ventricular failure.

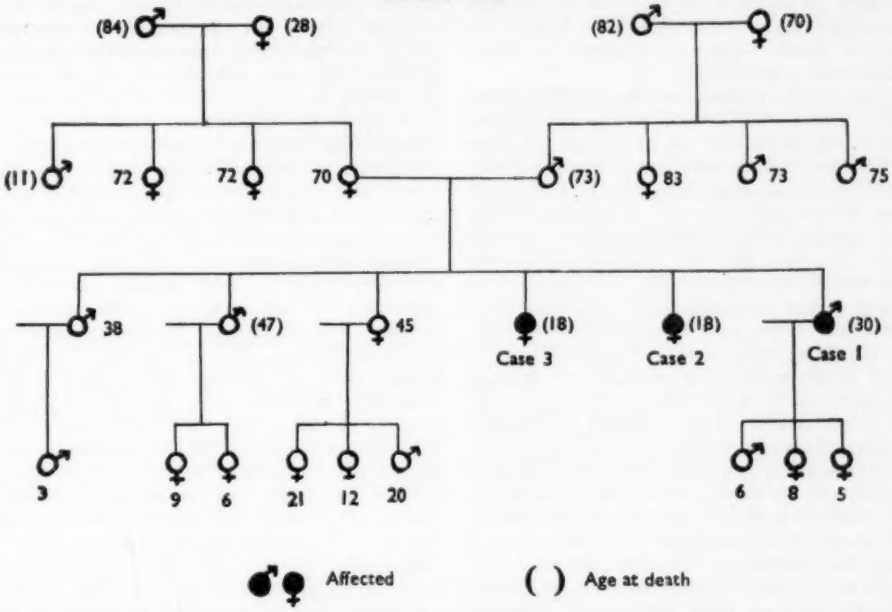
Case 3.—P. D., the sister of Cases 1 and 2, also died suddenly at the age of 18 years on December 26, 1939, exactly seven years before the death of her sister. In this case also there was no history of previous ill-health. She was dancing without distress until after midnight, when she returned home and retired to bed. A few minutes later her parents went in to see her and found her dead. Her case was also the subject of a coroner's inquiry, but copies of the post-mortem findings are no longer available although it appears from the records that the precise cause of death was never established

Other Relatives.—The antecedents of these three cases (Table II) on both male and female sides have in general been long-lived, but, of the exceptions, their mother's only brother died at the age of 11 years from pneumonia, and her mother died at the age of 28 years during childbirth. One of their brothers committed suicide but was known to be physically well. Their mother is an obese, arthritic woman, with a blood pressure of 170/100 mm. Hg, slight dyspnoea on exertion, and occasional swelling of the ankles. Radiographs of the chest showed no cardiac enlargement and an electrocardiogram was normal. The surviving siblings and all the nine members of the next generation, with the exception of one young man in the Navy, have been examined clinically and radiologically without finding any evidence of cardiomegaly. Electrocardiograms have been taken of five members of the family and show no abnormality.

Discussion

Study of the pathological appearances of the heart in these cases and in the eight previous necropsy reports referred to in Table I reveals no uniform pattern and indeed the variability of the post-mortem findings suggests that the cases listed here as examples of familial cardiomegaly may have had more than one cause. For descriptive purposes they may be separated into those in which the muscle hypertrophy was the main or only abnormal finding and those in which hypertrophy was accompanied by widespread interstitial fibrosis.

TABLE II
FAMILY TREE



Only two cases fall into the first category, namely, Cases 1 and 14 (Table I). In the former case the heart weighed 600 g. and was grossly dilated, being described by Dr. Tracy Mallory as "one of the largest hearts from the point of view of capacity that we have ever seen." All the chambers shared in the hypertrophy, but there was no other microscopic abnormality of the myocardium. In Campbell and Turner-Warwick's case the heart weighed 1,050 g., chiefly due to hypertrophy of the left ventricle which measured up to 25 mm. in thickness. Histologically there was simple hypertrophy of muscle fibres some of which showed central vacuolation; the nature of the vacuolar material was not established, but stains for fat and glycogen were negative.

The remaining cases belong to the second fibrotic group (Cases 2, 3, 4, 7, 9, and 12 in Table I and our own Case 1). Moderate or marked cardiac hypertrophy was present in all, the lowest heart weight recorded being 737 g. (Case 9) and the highest 1,134 g. (Case 4). Extensive myocardial fibrosis was present in all cases although the precise distribution of the fibrous tissue is not always recorded. In most the fibrosis affected both right and left ventricular walls, but in few was there any reference to the histological appearance of the auricles. In no case has there been found any macroscopic or microscopic evidence of disease of the coronary arteries. Occasional focal collections of chronic inflammatory cells have been noted in two cases (Cases 12 and 3) similar to the occasional foci present in the first case described here; Case 3 also showed patchy necrosis of muscle fibres similar to that occurring in our Case 1. Two of the cases in this fibrotic group (4 and 9) have shown unusually marked deposits of glycogen within the muscle fibres, although similar in other respects to the remainder of the group.

Consideration of these pathological types suggests a fundamental difference between those showing extensive myocardial fibrosis and those in which the pathological picture is one of simple hypertrophy. Despite the not dissimilar clinical features it seems unlikely that these are different stages of the same disease process or indeed that they have any aetiological relationship. The two types have not been found in the same family.

The picture of the fibrotic type appears to be that of replacement fibrosis and survival hypertrophy. Previous episodes of myocarditis which might account for this picture have naturally been sought in case histories. Rheumatic infection is recorded in only three cases (11, 12, and 13), in

two of which the diagnosis was doubtful ; Aschoff nodes were not found in the only case which came to necropsy. In the remaining cases there is no history of illness suggesting Fiedler's or other non-rheumatic myocarditis.

Paulley, Jones, Green, and Kane (1956) have, however, described several cases of fibrotic cardiomegaly which they attributed, on the basis of positive serological reactions, to toxoplasmosis. In one family, at least three of the four cases coming to necropsy showed a fibrotic cardiomegaly similar to that described above, although atypical in that the left ventricle was not predominantly affected and mural thrombus and embolism was a marked feature. Moreover, two of the four cases had enlarged firm spleens. Clinically their cases bore less resemblance to familial cardiomegaly, the absence of syncope being particularly noteworthy. Paulley *et al.* (1956) were unable to demonstrate toxoplasma bodies in the myocardium of their cases and positive serological reactions cannot be considered as conclusive evidence of active toxoplasma bodies from a case of obscure myocarditis in which evidence of generalized toxoplasmosis was lacking, but toxoplasma infection may well have been the aetiological factor in the cases of Paulley *et al.* In view of their findings we obtained samples of serum for toxoplasma antibody titration from a brother, son, and wife of our Case 1 and in no instance was a significant antibody titre recorded.

The finding of glycogen deposits in the muscle fibres in Cases 4 and 9 raises the question as to whether some cases of familial cardiomegaly may be due to a disorder of glycogen metabolism. In a few cases of Von Gierke's disease, glycogen infiltration has been confined to the heart, so-called "cardiomegalia glycogenica," but di Sant'Agnese, Andersen, and Mason (1950), who have recently reviewed this subject, consider that a fatal termination within the first year of life is invariable in this condition, and, moreover, those cases of glycogen storage disease in which the heart is involved do not progress to myocardial fibrosis. Russell (1948), in trying to assess the significance of the presence of muscle glycogen deposits in post-mortem tissue, examined specimens of muscle from 11 necropsy cases taken at varying intervals after death ; although she was able to demonstrate glycogen in the muscle fibres the infiltration was scanty compared with that found in the first case described by Evans (1949). The exact significance of these glycogen deposits is obscure, but the mere presence of glycogen within the myocardium does not provide a basis for the diagnosis of glycogen disease. In the light of our present knowledge it seems more reasonable to regard the deposits as a non-specific infiltration which may complicate the pathological pattern of the fibrotic form of familial cardiomegaly.

A further possible aetiological factor is the curious association between cardiomegaly and two familial neurological conditions, Friedreich's ataxia and progressive muscular dystrophy. The association of myocarditis with Friedreich's ataxia was first recorded by Pitt (1886–7). Several reports later appeared in the Continental literature and in 1946 Russell reported four examples of the condition with post-mortem details ; not infrequently the neurological lesion is accompanied by a chronic progressive myocarditis of fibrotic type which closely resembles that found in the fibrotic form of familial cardiomegaly. It was Russell's opinion that the heart muscle is destroyed through a focal, piecemeal, coagulative necrosis as a result of which the fibres are ultimately replaced by collagenous tissue, the surviving muscle undergoing a compensatory hypertrophy. This description could well be applied to the histological appearances in the cases of familial cardiomegaly now described, although, as Evans (1949) comments, "the relation of this condition to Friedreich's disease would be more definitely established if instances of each were met with in one family." Roth (1948) described a family affected by Friedreich's disease and peroneal muscular dystrophy in which there was a high incidence of heart disease, but there was no evidence to suggest that they were cases of familial cardiomegaly. Some 30 cases of cardiac involvement have been reported in progressive muscular dystrophy and the subject has recently been reviewed by Bevans (1945) and Storstein and Austarheim (1955). The post-mortem appearances of the heart in these cases seem to show a very variable picture ; sometimes the hearts are atrophic and in other cases marked hypertrophy is found. Macroscopically fatty infiltration and intracellular vacuolation of the fibres has been described ; in several cases a patchy fibrosis, often maximal in the outer myocardium, has been present. This association of hereditary neuropathies and fibrotic cardiomegaly has suggested to some that the cardiopathies in the familial and neuropathic varieties may have a common aetiology which may lie in an as yet undetected inborn error of metabolism. Support for this "chemical theory" of diffuse cardiac fibrosis is reinforced by the knowledge that diffuse

myocarditis may follow the ingestion of a variety of chemical compounds including arsenicals, sulphur, and sulphonamides (Elster, Horn, and Tuchman, 1955), and recently McAllen (1955) has observed severe myocardial changes, including widespread fibrosis, supervene in two cases in which there was prolonged depletion in potassium absorption. These human lesions were strikingly similar to the cardiac lesions found in potassium-deficient animals.

In conclusion it must be admitted that in spite of the numerous theories of origin, the aetiology of familial cardiomegaly still remains obscure.

Summary

A family with cardiomegaly is described in which the three affected members died suddenly when apparently in good health and were submitted to necropsy.

The clinical and pathological features of these and previously published cases of familial cardiomegaly are discussed and the possible aetiology of the condition considered.

Our thanks are due to Mr. L. G. Powell, H.M. Coroner for South Westmorland, for making available previous post-mortem reports.

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OBSERVATIONS ON A PATIENT WITH THE MALIGNANT CARCINOID SYNDROME

BY

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The syndrome of malignant carcinoid tumour with excessive production of 5-hydroxytryptamine (5-HT, serotonin, enteramine) is currently of great interest (Mattingly, 1956; MacDonald, 1956; Ritchie, 1956; Thorson, 1958). As well as reporting clinical, pharmacological, and necropsy data from a typical case, this paper describes for the first time changes in the pulmonary vasculature that may be relevant to the pulmonary hypertension occurring in some cases with carcinoid tumour.

Special attention has also been given to the fixation and staining of carcinoid tumours, and a new staining method is described which suggests that there is only one type of cell in these tumours.

Case Report

Mrs. C. W., aged 62, was admitted to the Royal Infirmary, Dundee, on November 19, 1956. For about one year she had had vague right upper abdominal pain, and for some six months had been losing weight (2 stone) and had had diarrhoea (average, three to four loose stools daily). She had had bleeding haemorrhoids for many years but no other illness of note.

On examination, she was rather obese and florid with marked venous telangiectasis of the face, but she stated that she came from rather florid stock and her appearance had hitherto not caused comment. Blood pressure was 175/95 mm. Hg. There was a mass in the right hypochondrium with tenderness and guarding over it. At laparotomy (November 22) a Meckel's diverticulum, containing a hard nodule, was removed and biopsy was taken of one of many metastatic deposits in the liver.

Histological Report (No. 2098/56).—Dr. W. W. Park reported that there were two specimens: (*a*) a Meckel's diverticulum, 3 cm. long and 2 cm. wide, containing in the wall at its distal end a firm oval nodule 6 mm. in diameter and of a grey-white colour on gross section, and (*b*) a narrow wedge of firm grey-white (liver) tissue. Sections showed the following features.

Nodule in Diverticulum.—This is an argentaffin carcinoma (carcinoid tumour) spreading widely into the muscular coats of the diverticulum and through them into the subserosa. The growth shows an unusually glandular structure.

Liver Biopsy. — This shows almost complete replacement of liver parenchyma by argentaffin carcinoma, with the same unusually glandiform structure.

The patient made a good recovery and went home on December 11, but her right upper abdominal discomfort continued and she returned to hospital on March 11, 1957. It was found that these symptoms could be largely relieved by codeine and she was discharged home.

Cardiac Catheterization.—The patient and her relatives were most co-operative, and after the procedure had been fully discussed with them the patient volunteered for this procedure, which was carried out uneventfully on March 21.

Haemodynamic studies showed no abnormality. Pressure pulse patterns were normal. Pressures were 22/10, 22/0, and 0 mm. Hg in the pulmonary artery, right ventricle, and right atrium respectively. There was, therefore, no pulmonary hypertension and no demonstrable stenosis or incompetence of pulmonary or tricuspid valves. No unusual pattern was seen at routine intracardiac electrocardiography.

Subsequent Progress. — The patient enjoyed reasonable health for some months, but the liver continued to enlarge and she needed increasing amounts of codeine for relief of pain. In August, 1957, the pain and diarrhoea increased, she became weak and anorexic, and she was admitted to hospital for the last time on September 5, 1957. She was still well nourished but extremely lethargic. The liver had grown much larger since her last admission. Blood pressure was 150/75 mm. Hg. There was still no evidence of disease in the cardiovascular and respiratory systems. She had rather a sore red tongue and throat but no skin rash suggestive of pellagra, which has been reported as complicating this syndrome (Bridges, Gibson, Loughridge, and Montgomery, 1957). She went progressively downhill and died on September 9.

Chemical Studies

These studies were made during the last nine months of the patient's life, and after her death on tissues obtained at necropsy.

Methods.—These were as follows:

(1) The 5-H was extracted from blood, plasma, and necropsy tissue with acetone, evacuated to dryness *in vacuo*, and taken up in saline solution for biological assay on the atropinized oestrous uterus of the rat. Extracts were retested in the presence of the specific 5-HT antagonist, lysergic acid diethylamide.

(2) For chromatography, spots of extracts from the organic phase together with reference markers of 5-HT creatinine sulphate and 5-hydroxyindoleacetic acid (5-HIAA) were placed on Whatman No. 4 filter paper, run ascending in butanol-acetic-acid-water (4:5:1) overnight, dried, and developed with Ehrlich's reagent.

(3) The 5-HIAA in urine was estimated by the method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus, and Smith (1956).

(4) The histamine contents of blood and urine were estimated for us by Dr. R. G. Mitchell, Department of Paediatrics, University of St. Andrews, using Code's (1937) modification of the Barsoum-Gaddum method for determining histamine in blood, and the method of Roberts and Adam (1950) for histamine in urine.

The blood samples were drawn from peripheral veins and separated within minutes. Those of January 21, 1957, and January 28, 1957, were obtained in siliconed syringes, but the sample of September 7, 1957, was obtained in a dry syringe. The platelet layers and supernatant plasmas of the siliconed samples of January 21, 1957, and January 28, 1957, were extracted separately; the 5-HT content was wholly in the platelet layers, there being none in the plasma. The sample of September 7 was platelet-free, the platelets having presumably ruptured during sampling and released their 5-HT content into the plasma.

At cardiac catheterization, despite the use of a nylon catheter and siliconed syringes, all blood samples were devoid of platelets.

Urine was collected over 24 hours in a bottle containing 25 ml. glacial acetic acid and 3 ml. toluene.

Results.—The results of the estimations of 5-HT in blood and 5-HIAA in urine are shown in Tables I and II. The 5-HT content of various tissues was assayed *post mortem*, and the results are shown in Table III. The hepatic and cardiac nodules were surrounded by normal tissue and the ratio of normal tissue to metastatic carcinoid, in the pieces of tissue analysed, was not known.

The histamine level in the blood on January 21 was 0.06 μg./ml., the basophil count being 63/c.mm. and the eosinophil count 238/c.mm. During the preceding 24 hours urine had been collected for histamine determination, and its content of free histamine was found to be 8.3 μg./24 hours. Dr. Mitchell regards the values for blood and urine as well within the range of normal for healthy adults (Mitchell and Code, 1954; Mitchell, Butt, and Code, 1954).

Comparison of our figures for 5-HT and 5-HIAA with those of Pernow and Waldenström (1957) shows that our case is typical of the functioning malignant carcinoid syndrome (Table IV).

TABLE I

RESULTS OF ESTIMATIONS OF 5-HT IN BLOOD AND 5-HIAA IN URINE

Date	Blood Content of 5-HT (μg. per ml.)	Urinary Content of 5-HIAA (mg. per 24-hour Output)
25/11/56	—	160
30/11/56	—	204
21/1/57	0·133 (whole blood)	96
28/1/57	0·24 (,, ,,)	—
7/9/57	0·25 (platelet-free plasma)	300

TABLE II

PLASMA CONTENT OF 5-HT AFTER CARDIAC CATHETERIZATION

Site of Sample	5-HT (μg. per ml. of Platelet-free Plasma)
Right pulmonary artery	0·241
Mid-right atrium	0·225
Left hepatic vein	0·242
Right hepatic vein	0·250
Inferior vena cava	0·162
Brachial artery	0·192

TABLE III

ASSAY OF 5-HT CONTENT OF POST-MORTEM TISSUE

Tissue	5-HT (μg. per g. of Tissue)
Ileum, adjacent to operative resection	2·77
Kidney	0·46
Brain (hypothalamus)	1·6
Lung*	17
Left ventricular myocardium	4
Metastatic nodule in left ventricle	88
Hepatic metastasis*	60
Lymph node metastasis*	2,000

* 5-HT content was confirmed by paper chromatography; on each paper a trace of 5-HIAA seen, but no evidence of 5-hydroxytryptophan (5-HTP) in any.

TABLE IV

COMPARISON OF VALUES FOR 5-HT AND 5-HIAA IN PERNOW AND WALDENSTRÖM'S (1957) AND PRESENT CASES

	Normal	Carcinoid Syndrome	Present Case
Serum 5-HT (μg./ml.)	0·03 to 0·2	0·2 to 0·6	0·133 to 0·25
Urine 5-HIAA (mg./24 hours)	2 to 10	60 to 2,000	96 to 300

Moreover, at necropsy the 5-HT content in a lymph node metastasis was 2 mg. per g. of tissue, comparable to the findings in other cases (Lembeck, 1953; Sjoerdsma, Weissbach, Terry, and Udenfriend, 1957).

Blood 5-HT Content from Different Vascular Sites.—Goble, Hay, and Sandler (1955), studying a case of carcinoid syndrome, found that the free plasma 5-HT (as distinct from platelet-bound 5-HT) was reduced in amount during its passage through the lungs; they attribute this to the removal of 5-HT by the enzyme mono-amine oxidase in the lungs in high concentration. In cardiac catheterization of six patients with the carcinoid syndrome, Sjoerdsma and his colleagues (1956, 1957) failed to confirm this finding. They stress the difficulty of separating plasma from formed elements and state that proof of pulmonary arteriovenous 5-HT difference must await the elaboration of more reliable methods. Our patient had been found earlier in the course of her illness to have no free plasma 5-HT (Table I). At cardiac catheterization, two months later, blood was withdrawn from several vascular sites (Table II). These samples were poor in platelets or else there were none; thus the plasma content of 5-HT could be attributed largely or entirely to rupture of platelets during sampling. There was no gross difference between the 5-HT content of the hepatic venous, pulmonary arterial, and brachial arterial blood samples. In view of the complicating views mentioned above and the technical difficulties involved, it is obviously desirable to pursue such catheterization studies if clinically justifiable once technical methods have been improved. In the present case, there were numerous metastatic deposits both in the lungs and in the left ventricular wall; these could well have been contributing 5-HT directly into the greater circulation and thereby eliminated any evidence of destruction of 5-HT in the pulmonary traverse.

Histamine Excretion.—Some cases of the carcinoid syndrome have increased blood levels and urinary excretion of histamine, the excess of 5-HT perhaps producing secondary release of histamine (Pernow and Waldenström, 1957). Our patient, however, had normal levels of blood and urinary histamine.

Necropsy (N/2877/57)

There was no abnormality of the head and neck. A small effusion was present in each pleural cavity. The lungs (right 270 g., left 225 g.) appeared normal.

In the heart (350 g.) there were three nodules of pale tumour tissue within the myocardium of the left ventricle. There was no ventricular hypertrophy and the heart valves were healthy apart from slight fibrous thickening at the base of the pulmonary valve, which was not stenosed and was completely competent.

There were a few peritoneal adhesions over the upper surface of the enlarged liver (2,950 g.), but no peritoneal effusion. The liver substance was almost totally replaced by yellowish-brown tumour tissue as were all the lymph nodes in the porta hepatis. Thirty centimetres proximal to the ileocaecal valve there were numerous adhesions at the site of the surgical resection. The bowel lumen, however, was fully patent and there was no evidence of tumour here or in any other portion of the gastro-intestinal tract, including the appendix.

Small nodules of metastatic tumour were found in the otherwise normal-looking spleen, pancreas, and kidneys. The uterus was enlarged to twice its normal size by several intramural and subserous fibroids, but was free from secondary deposits.

Histology of the Tissues.—The tissues were fixed by the standard method of 10% formol saline followed by formol corrosive, saturated corrosive, dehydration, and paraffin embedding (Lendrum, 1951).

All sections of the lungs show numerous secondary nodules, many consisting of only a few cells, distributed in random fashion with no constant relation to blood vessels or lymphatics. Metastases are also present in the left ventricle (Fig. 1), both kidneys, liver, lymph nodes, and spleen. Mitoses are rare.

The pulmonary valve appears to be within normal limits and Weigert's stain for elastica shows the usual elastica pattern without any of the changes noted by MacDonald and Robbins (1957) in carcinoid fibrostenosis.

Sections were stained by the diazonium dye fast red B (Pearse, 1953) and by Davenport's modification of Bodian's protargol procedure in an attempt to demonstrate the so-called "argentaffin" and "argyrophil" cells. Argentaffin cells are said by Jacobson (1957) to show red or reddish-brown granules with the diazonium method and black granules with both Masson-Fontana and with Davenport's method, whereas "argyrophil" cells react only with Davenport's method. In our case, the Davenport method shows black or brown granules in nearly every cell which on the basis of other methods is not autolysed; the diazo-positive cells, on the other

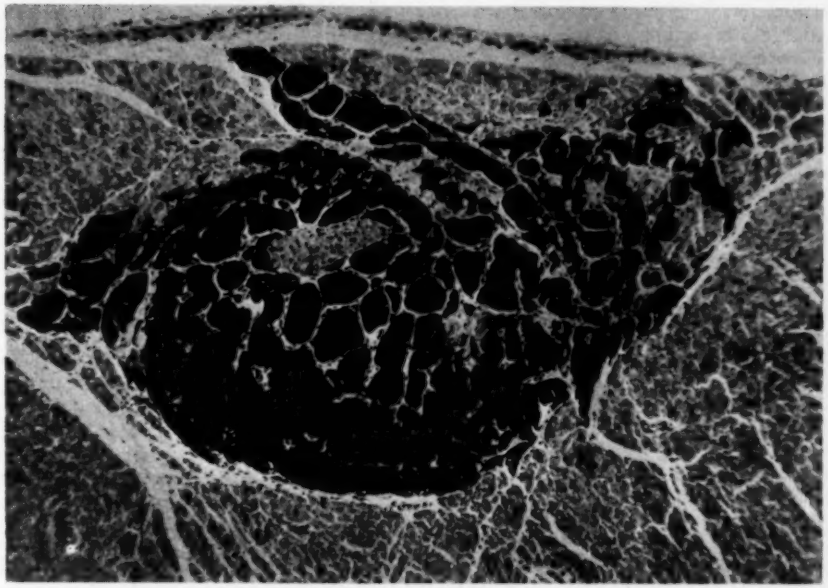

FIG. 1.—Left ventricle, × 75. The phloxinophilia of the tumour cells in this metastasis shows up darkly, thanks to a minus-red filter. Note the occasional acinar formation. Stained: phloxin, "lissamine" flavine (Dundee 58-A). Present case.

hand, are scanty, being confined to the periphery of large masses of cells or to groups of only a few cells.

Sections from the various metastases were also stained by a method recently elaborated by Lendrum and Fraser (see Appendix). This modification of the phloxin-tartrazine method (Lendrum, 1939, 1947) has now been tested by these workers on over 50 examples of carcinoid, and the following paragraphs are a joint statement based on the conclusions they have already reached.

The method shows in well-fixed tissue an aggregation of strongly red (phloxinophilic) granules at the basal, subnuclear part of those tumour cells that lie at the periphery of tumour masses. Towards the centre of the larger masses and also gradually as one moves in from the surface of the block, the granules disappear, being replaced, as it were temporarily, by a structureless red staining of the cytoplasm. This gradation of preservation of the granules is related in part to inadequate penetration even with the best fixative (formol corrosive). The poorer the fixation, as measured by cytological definition and tinctorial specificity with other stains, the fewer cells are seen with sharply defined phloxinophilic granules. Fresh tissue in suitably small blocks if well fixed either by formalin, which happens sometimes, or by the more consistent formol-corrosive will show equally good phloxinophilic demonstration of the granules. Apart from fixation, the absence of granules and even of diffuse phloxinophilia in the centre of the larger masses of tumour cells is largely the result of degenerative change, as occurs in the masses of encephaloid carcinoma; comparable change is also seen in post-mortem material and the loss is most obvious in tissue from the bowel wall and hepatic metastases. These autolytic changes are accompanied by deterioration in the quality of the nuclear and other staining.

In the adenomatoid areas, which in the present case were particularly obvious in the hepatic and renal metastases, the lumen commonly contained a circular mass which was strongly P.A.S. positive and also showed an extreme retention of phloxin, even in those parts where the tumour cells were so autolysed that they had lost all phloxinophilia.

The Pulmonary Vasculature.—Fifteen blocks of lung were fixed, three from each of the five major lobes. Sections were stained by Weigert's elastica method and the arterioles (vessels less than 100 μ in diameter) were measured with the aid of an

ocular micrometer. The same various diameters were measured on sections, similarly prepared, from five normal controls, one case of severe mitral stenosis, and a case of primary pulmonary hypertension.

The Measurements. — Various authors—Valenzuela, Toriello, and Thomas (1954), Goodale and Thomas (1954), Denst, Edwards, Neubuerger, and Blount (1954), O'Neal, Thomas, and Hartroft (1955), and Heath and Whitaker (1956)—have given their histological criteria for a diagnosis of pulmonary hypertension, but they are by no means agreed on a common method. Valenzuela *et al.*, Goodale *et al.*, and Heath *et al.*, however, are agreed that pulmonary vessels whose external diameter is less than 100 μ should only have one layer of elastica, no media, and a wall thickness less than 15% of the external diameter.

For the purpose of this investigation measurements were made of the external diameter, wall thickness, and lumen diameter in all cases and finally expressed as a percentage ratio between the wall thickness and the external diameter. Only vessels cut in cross-section or near cross-section were measured, all measurements being made in the same diameter. When the vessel wall varied in thickness, at least two separate measurements were made in different diameters of what was considered to be a representative section of the wall. Measurement of the vessel lumen was used merely to confirm the accuracy or otherwise of the wall thickness and external diameter.

Such an estimation at least allows a comparison to be made between the pulmonary vessels of the control cases and those of the present case of malignant carcinoid, although our findings in this latter might, to some authors, be not indicative of pulmonary hypertension.

Results.—In the normal control lungs 100 vessels were measured; 34 vessels had a wall thickness external diameter less than 10%, 52 vessels were between 10 and 15%, nine vessels were between 16 and 20%, and five vessels between 21 and 25%. In the case of severe mitral stenosis a shift to the right was noted; one vessel less than 10%, nine between 10 and 15%, 18 between 16 and 20%, three between 21 and 25%, and five between 26 and 30%. A similar picture was seen in the case of primary pulmonary hypertension; of 84 suitable vessels, seven were less than 10%, 46 between 10 and 15%, 19 between 16 and 20%, eight between 21 and 25%, two between 26 and 30%, and two greater than 30% (Figs. 2, 3, and 4).

In the case of malignant carcinoid, not only was there a shift to the right, but there was also a wider scatter of results with considerable flattening of the scale. Eighty-five vessels were suitable for measurement; 19 were less than 10%, 24 between 10 and 15%, 24 between 16 and 20%, 10 between 21 and 25%, and eight between 25 and 50% (Fig. 5).

Study of the lobar distribution of the vascular changes showed that the changes were more severe in the right lung (Table V), and, although

TABLE V
DISTRIBUTION OF VASCULAR CHANGES AS RATIO OF WALL THICKNESS TO DIAMETER

N 2877 Carcinoid	No. of Vessels	10%	10–15%	16–20%	21–25%	26–50%
Right lung	53	7	16	14	9	7
Left ,,	32	12	8	10	1	1

the number of vessels suitable for measurement in the sections from any given lobe was rather small, it was none the less possible to state with fair assurance that the change was more prominent in the right upper lobe than in the right lower lobe.

We make these observations on the zonal differences without comment, except to say that these arteriolar changes may have no relation to the narrowing described by Harrison (1958); in his studies on post-mortem angiography he noted no difference between the lungs, nor between the lobes in primary pulmonary hypertension, whereas the narrowing was distinctly more intense in the lower lobes in mitral stenosis.

The recognition microscopically of pulmonary hypertension is undoubtedly possible, but we believe that the degree of pulmonary hypertension can only be assessed accurately after the laborious method of measuring suitable vessels and comparison with control material prepared in a similar manner.

Since the lungs in all eight cases were prepared in a fairly comparable way it seems reasonable to believe that any fallacies due to artefact would cancel out. Further, the similarity of the control cases as a group and their statistically significant difference from the two hypertensive cases suggest that the measurements provide objective information. Finally, the facts observed in the carcinoid material would seem to indicate that the mechanism of pulmonary hypertension had been set afoot there.

Discussion

In 1888 Lubarsch described a primary tumour of small bowel and appendix which he called a

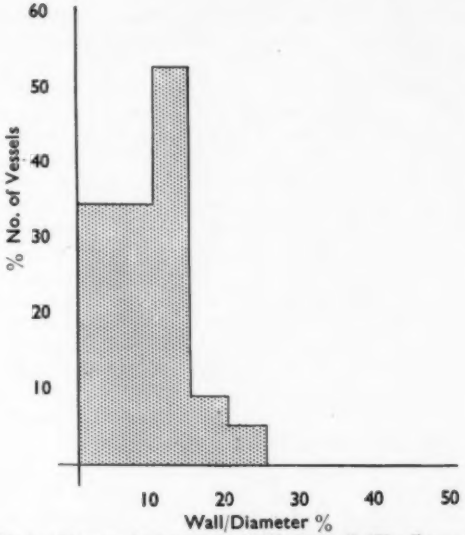

FIG. 2.—Diagram of measurements of 100 vessels, 40–100 μ diameter, end-on, from five control cases as follows:

Age	Sex	Diagnosis
36	M	Fractured skull
63	F	Anorexia nervosa
67	F	Carcinoma cervix
69	M	Myocardial infarction
89	F	Fractured skull

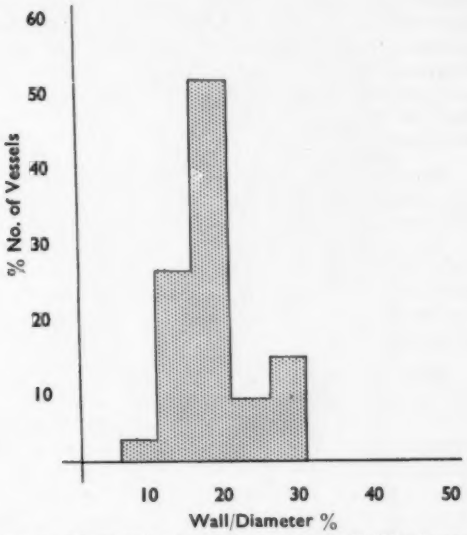

FIG. 3.—Diagram of measurements of 36 vessels, 40–100 μ diameter, end-on, in a case of severe mitral stenosis in a man aged 57.

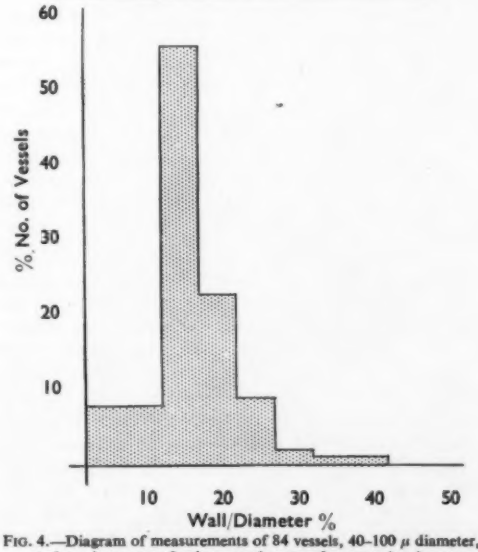

FIG. 4.—Diagram of measurements of 84 vessels, 40–100 μ diameter, end-on, in a case of primary pulmonary hypertension in a man aged 34.

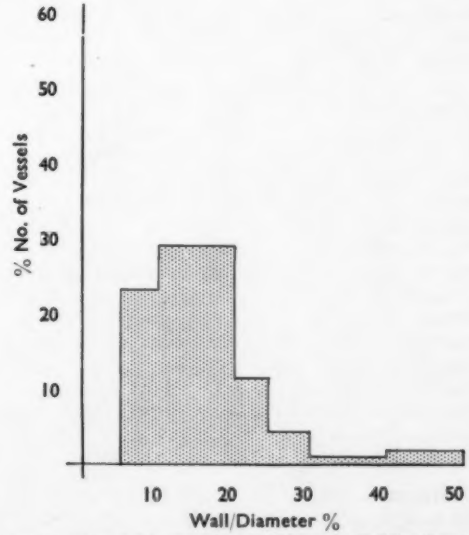

FIG. 5.—Diagram of measurements of 85 vessels, 40–100 μ diameter, end-on, from 15 different sections in a case of malignant carcinoid in a woman aged 62.

" little carcinoma," later termed " carcinoid " by Oberndorfer (1907). Pierre Masson showed in 1914 that the tumour contained intracytoplasmic granules which reduced silver salts, and he proposed the name " argentaffin tumour." He also postulated an endocrine function which at last he has had the satisfaction of seeing confirmed.

Site of Primary Tumour.—The primary tumour was situated in a Meckel's diverticulum, one of the rarer sites. MacDonald found nine such cases in the literature and added two cases from his own series of 140 extra-appendiceal carcinoid tumours. Another case has since been described by Smith and Campbell (1956), which with the present case brings the total to 13.

Haemodynamic Data.—In this patient, as in two of the three cases studied by Sjoerdsma *et al.* (1956), there were no abnormal findings at catheterization of the right heart. In the cases of Biörck, Axén, and Thorson (1952) and Goble, Hay, Hudson, and Sandler (1956) there were abnormal right heart pressure recordings due to valvular disease.

Early in the study of the malignant carcinoid syndrome it had been guessed that a cause of right heart disease might be pulmonary arteriolar constriction by 5-HT, thereby raising the pulmonary vascular resistance (Thorson, Biörck, Björkman, and Waldenström, 1954 ; Jenkins and Butcher, 1955). This suggestion was refuted by Goble *et al.* (1956), who showed normal pulmonary vascular resistance even during flushing, when circulating 5 HT was in fact increased to a very high level. The following year, Sjoerdsma *et al.* (1957) reported an example of raised pulmonary artery pressure (40/20 mm. Hg) ; their patient had suffered frequent asthmatic attacks, attributable to 5-HT, but there is no real evidence that the raised pulmonary arterial pressure was not merely due to his chronic pulmonary damage.

Clinically our patient showed the most constant feature of the syndrome, namely, chronic diarrhoea. She had a pronounced flush, but its diagnostic value was uncertain since she stated that other members of her family were also florid. She had no clinical evidence of the less common features such as acquired cardiac disease, bronchospasm, or arthropathy (Sjoerdsma *et al.*, 1956).

Histochemistry.—Special attention was paid to the staining properties of the tumour cells because of the suggestion by Hamperl (1952) and Jacobson (1957) that there are two types of tumour cell, argentaffin and argyrophil (Hamperl and Lattes, 1957). Since then, Sandler and Snow (1958) have suggested that argentaffin cells secrete 5-hydroxytryptamine (5-HT) and argyrophil cells secrete 5-hydroxytryptophan (5-HTP). They described three unusual cases of atypical malignant carcinoid syndrome (one of their own, and two quoted from the literature) in which there was patchy flushing attenuated by antihistamine drugs. The tumour cells lacked argentaffin granules and examination of the urine showed excretion of 5-HTP and greatly increased excretion of 5-HT and histamine. They guess that tumour tissue may in future be found to contain both argentaffin and argyrophil cells in varying concentrations and that both 5-HT and 5-HTP may be secreted by the tumours. In our case no 5-HTP was found at necropsy although the tumour contained a predominance of argyrophil cells, the argentaffin cells being extremely scanty.

The granules of enterochromaffin cells (Kultschitzky, 1897) in the bowel and in the carcinoid tumours studied are easily and effectively demonstrated by the new method of Lendrum and Fraser if cytological preservation is good. The granules in many of the cells also give a positive although much less obvious reaction with the diazo method, with Schmorl's method as modified by Laskey and Greco (1948), and with Verhoeff's stain for elastica. In the present case, tissues were fixed within four hours of death and excellent phloxinophilia of the granules was found in the cardiac, pulmonary, renal, lymph node, and splenic metastases. The hepatic nodules were more autolysed and gave poorer results with the new method and almost negative results with the Davenport technique, but this latter finding has to be interpreted with caution because Lendrum and Fraser have found that although formol-corrosive is ideal as a fixative for the new method it does partially inhibit both the silver methods.

Comparison of the various staining methods (including the silver methods) and the various fixatives used, with particular note of the cytological quality of the sections, leads us to the conclusion that the new method is more consistently informative than the silver impregnations—the silver proteinate (Davenport and variants) and the Fontana-Masson. Probably the Fontana-positive cells (argentaffin) occurring in human tumours all also react with proteinate ; the other proteinate-positive cells, those that fail to react with Fontana, are described as argyrophils. Thus the less efficiently the Fontana method works, the greater the proportion of the tumour cells that have to be accepted as

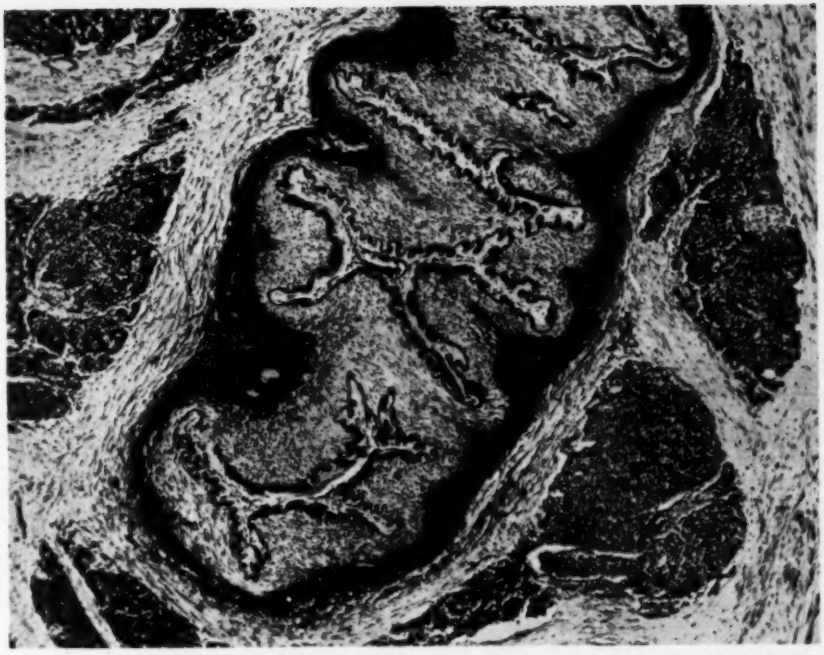

FIG. 6.—Artery, × 45. The elastica of this vessel from near an ileal carcinoid shows an intense phloxinophilia. Note the phloxinophilia of the adjacent tumour tissue. Stained: phloxin, "lissamine" flavine (Dundee 58-A). Minus-red filter. Newcastle case 57/5318.

FIG. 7.—Artery, × 45. This is an adjacent section to show that the phloxinophil material in the last is in fact elastica. The Verhoeff stain gives a slight reaction with the granules of the tumour cells. Stained: Verhoeff and "lissamine" flavine.

argyrophil cells. From our studies it seems clear that autolysis, poor formalin fixation, or the use of formol-corrosive (mercurial) fixation all reduce the granule content of the tumour cells as measured by the Fontana method. We, therefore, hesitate to accept any tumour as an argyrophilic tumour unless the authors accompany their assessment by proof of perfect fixation, and we would suggest that the chemical observations on 5-hydroxytryptophan excretion in the urine (Sandler and Snow, 1958) may be indicative of autolysis occurring within the metastases. Our morphological observations do not justify us in thinking that there is more than one kind of carcinoid tumour.

In their study of the series of carcinoids, Lendrum and Fraser have found an extraordinary phloxinophilia in the elastic tissue which in the region adjacent to the tumour is increased in amount. In five cases there is not only an increase of stromal elastica but also a quite unusual amount and distribution of elastic tissue in large arteries (Figs. 6 and 7). This curious alteration raised the speculation that some product of the tumour has a directly stimulating effect on elastic tissue, and consequently attention was turned to the state of the elastic tissue in the pulmonary arterioles in the present case.

The Pulmonary Changes.—The possibility was envisaged that the circulating 5-HT might act on the elastica of the pulmonary vessels and thus be the first link in the chain of causation of the pulmonary hypertension. If in addition there were metastases in the lung it might be possible to observe more notable effects on the vessels adjacent to the metastases. From the microscopical study of the lung sections we thought that the interstitial elastica was in fact more obvious, indeed increased, around the larger pulmonary metastases. Moreover, some of the smaller pulmonary arteries adjacent to the metastases showed an apparent increase of elastica with splitting of the laminae and there were many large intimal proliferations which considerably reduced the vessel lumen.

Further, the variation in the changes in the arterioles measured, shown by the wide base of the hypertensive index graph, points to local effects even if it does not exclude a general effect. Unfortunately the reconstruction necessary to confirm these local effects was not possible on the 15 random blocks we had.

It is perhaps important to distinguish the two sets of observations on the carcinoid case. Firstly we have the generalized and focal elastosis in the stroma and vessels of the lung, a finding that is difficult to measure satisfactorily but one whose validity is strengthened by its similarity to the changes occurring near carcinoid tumour elsewhere, for example, around the primary in five of Lendrum and Fraser's cases of ileal carcinoid. Secondly we have the hypertensive index graph, based on absolute measurements, with its obvious abnormality and partial resemblance to the graphs obtained from the lungs from mitral stenosis and primary pulmonary hypertension.

It is commonly held that the structural abnormalities found in the pulmonary vasculature of these last two conditions are the result and not the cause of the hypertension ; this view may be more emphatic than accurate even though its converse be untrue. Certainly our patient, six months before death, had a pulmonary artery pressure of only 22/10 mm. Hg, and to clinical observation she never provided evidence of pulmonary hypertension. Even at necropsy the only relevant feature was a slight fibrous thickening at the base of the pulmonic valve. Thus there was not enough pulmonary hypertension to provide us with obvious evidence of its existence, but there are changes in the lung that could reasonably be accepted as the result of pulmonary hypertension, and there is an elastosis that could be due to the products of carcinoid tumour. It is surely tempting to think that the elastosis is how the pulmonary haemodynamics are first upset in the carcinoid syndrome.

Summary

A case is described of functioning malignant carcinoid with clinical, haemodynamic, pharmacological, and necropsy data.

A new method is described for staining carcinoid tumour cells ; a wide study of these tumours suggests that there is only one type of neoplastic cell.

Histological changes have been observed in the lung and its arterioles that may be a clue to the pulmonary hypertension of the carcinoid syndrome.

We are indebted to Dr. D. Emslie-Smith for his help at cardiac catheterization, Messrs. D. S. Fraser and W. Slidders for considerable technical assistance, Mrs. D. Kirkland for Figs. 2 to 5, and particularly Professor A. C. Lendrum for his encouragement and help with the manuscript.

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APPENDIX

The following red and yellow stain (Dundee 58-A) prepared by the method of A. C. Lendrum and D. S. Fraser was used on sections from the various metastases.

Stain with haemalum, etc., as usual.

Phloxin 0.5% in water plus 0.5% calcium chloride —30 min.

Rinse in cellosolve and then rinse in deodorized domestic kerosene (Aladdin Pink paraffin of Messrs. Shell-Mex).

Leave overnight in jar of kerosene.

Rinse in cellosolve and immerse in jar of saturated solution of "lissamine" flavine FFS (C.I. 56205, Messrs. I.C.I.) in cellosolve.

Differentiation is adequate in three to four hours in well-fixed tissue, but sections of poor tissue may have to be taken out before the erythrocytes or even all the collagen have lost their red.

Rinse in cellosolve, in xylene, and mount.

Professor Lendrum acknowledges gratefully material from J. H. Biggart, A. C. P. Campbell, D. F. Cappell, A. Dick, J. S. Faulds, B. C. Morson, A. G. E. Pearse, J. H. Prain, G. H. Smith, and J. S. Young.

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TECHNICAL METHODS

TRIS Buffer for the Demonstration of Haemoglobin A_2 by Paper Electrophoresis

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(RECEIVED FOR PUBLICATION APRIL 30, 1959)

Trishydroxymethylaminomethane (TRIS) buffer has been recommended for finer separation of serum proteins in paper electrophoresis (Aronsson and Grönwall, 1957). We have employed it for the examination of haemoglobin variants and have found that, with a slight modification, it is suitable for this purpose. When used with a vertical tank the fractions do not move as far from the point of application as they do with barbiturate buffer (*p*H 8.6), but they are more sharply defined and the bands are narrower. In view of this it is possible to recognize, with the naked eye, small fractions which would escape discovery on paper electrophoresis with barbiturate buffer. This is particularly useful for the examination of specimens for haemoglobin A_2. Even normal proportions of haemoglobin A_2 are easily visible, and abnormal proportions of haemoglobin A_2 are recognized at once. By this technique the non-haemoglobin protein X of Derrien (1959), which is present in some haemolysates, separates clearly from haemoglobin A_2, and on staining the strips and eluting the protein dye complex the risk of inaccuracy due to the admixture of protein X to haemoglobin A_2 is avoided.

In Fig. 1 a haemoglobin specimen from a patient with haemoglobin H disease is compared with that from a case of thalassaemia minor. Fig. 1a is a photograph of the unstained strip and shows the absence of haemoglobin A_2 in the specimen of haemoglobin H disease. Fig. 1b shows the strip after staining with "light-green," and it will be noted that the X fraction is now visible, and that it is well separated from haemoglobin A_2.

Fig. 2 shows a photograph of an unstained strip of the electrophoretic pattern of a haemoglobin from a normal person and of a haemoglobin from a case

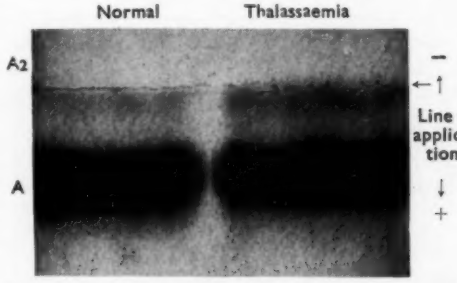

FIG. 2.—Unstained strip showing after electrophoresis haemoglobin A_2 just visible in the normal specimen and increased in thalassaemia.

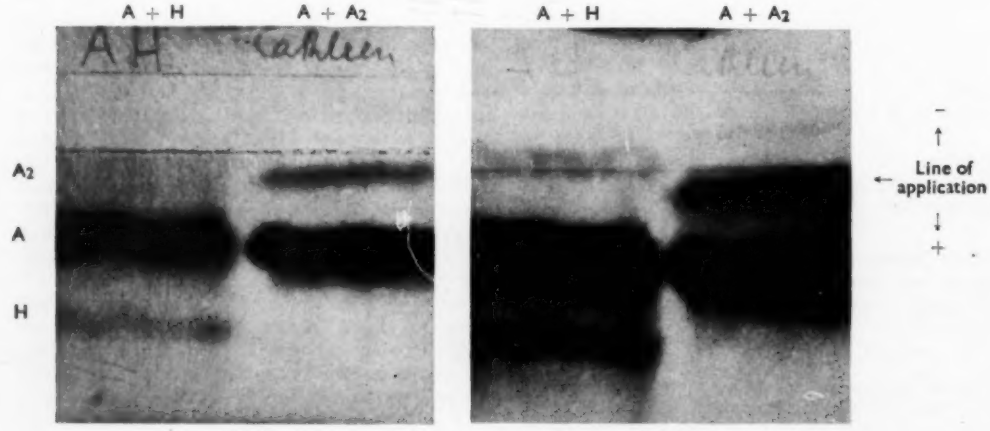

FIG. 1a

FIG. 1b

FIG. 1a.—Unstained strip showing haemoglobins A and H on the left and haemoglobins A and A_2 on the right.

FIG. 1b.—Strip stained for protein with "light green." Protein X is now visible in both specimens. On the right it is well separated from A_2.

of thalassaemia minor with an increased haemoglobin A_2 fraction.

The electrophoresis was carried out for 16 hours by the hanging strip method, using Whatman No. 3 paper for chromatography at 160 v., with a strip 8.5 cm. wide and 36.5 cm. long. The current per strip was 3.5 mA.

The slight but important modification of the TRIS buffer as given by Aronsson and Grönwall (1957) consists in using it more dilute, and the recommended preparation of the TRIS buffer is as follows:

Trishydroxymethylaminomethane	50.4 g.
Ethylenediaminetetracetic acid	5.0 g.
Boric acid	3.8 g.

Dissolve to a final volume of 1,000 ml. in distilled water

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A Technique for Electrophoresis in Agar Gel

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(RECEIVED FOR PUBLICATION OCTOBER 3, 1958)

Gordon, Keil, and Sebesta (1949) described the electrophoretic separation of proteins in agar gel. Grabar and Williams (1955) developed the method for the immuno-electrophoretic analysis of proteins in agar, and that technique is finding increasing application in the characterization of proteins. Uriel (1958a) has described how the use of electrophoresis in agar combined with immunological and chemical reactions allows a triple characterization of constituents of biological fluids to be determined, namely, electrophoretic mobility, immuno-chemical specificity, and chemical nature. Recently Uriel (1958b) has described the quantitative analysis of serum proteins after separation in agar gel. This paper describes how an electrophoresis box designed for free horizontal paper electrophoresis may be easily adapted for electrophoresis in agar gel.

Method

Buffer.—The buffer is *p*H 8.6 barbitone buffer ionic strength 0.05; sodium barbitone 5 g., hydrated sodium acetate 3.33 g., 0.1 N sulphuric acid, 34.2 ml., and water to 1 litre.

Agar Gel.—One per cent. agar (Davis Standard) was made up in buffer solution by heating in a water bath. While still hot the resulting solution was filtered through glass wool, poured on to a perspex plate and allowed to cool. The thickness of the agar sheet was 3–4 mm. The gel was cut into strips 25 cm. long by 4.5 cm. wide. For convenience several strips were prepared at a time and stored in a flat-bottomed enamel tray, under buffer solution, until required. On being removed from the tray and blotted, the strips were ready for immediate use. Uniform runs were obtained in gels stored in this way. It was usual to prepare a week's supply at a time.

Adaptation of the Electrophoresis Box.—A bridge of perspex was made by cutting a section from a sheet of perspex (6 mm. thickness), and this rested on the sides of the buffer solution compartments.

Gel : Buffer Solution Contact.—To eliminate the difficulties encountered in the various paper or gauze wick contacts between the agar gel and the buffer solution, it was so arranged that the gel itself dipped into the buffer solution, as shown in Fig. 1. This procedure was easily carried out and was found to provide a good gel : buffer contact. The agar gel is placed on a piece of celluloid (used *x*-ray film), folded

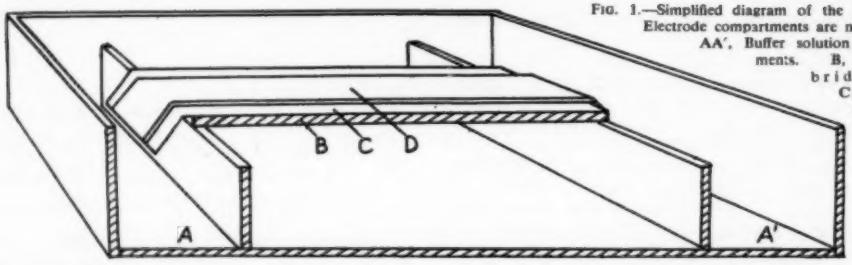

FIG. 1.—Simplified diagram of the apparatus. Electrode compartments are not shown. AA', Buffer solution compartments. B, Perspex bridge. C, Celluloid film. D, agar gel.

as indicated. This rests on the bridge and prevents any break where the gel dips into the buffer compartment. A second piece of film, lightly smeared with mineral oil, is placed on the surface of the agar gel while electrophoresis is in progress to prevent evaporation from the surface of the gel. This greatly reduces condensation and the formation of water droplets on the glass lid of the box. Without this precaution drops of water were liable to fall on to the gel during electrophoresis.

Application of Serum and Voltage for Electrophoresis.—A trough is cut in the centre of the gel strip. The serum is mixed with an equal volume of buffered 2% agar. While still warm the liquid is placed in the trough and allowed to set. Up to 0.1 ml. of serum may be applied in this way.

It was convenient to run electrophoresis overnight (17 or 18 hours) at 2 volts per cm. length of the agar strip in order to obtain a good separation of serum proteins. The current remained practically constant throughout the run. Fig. 2 shows a typical serum

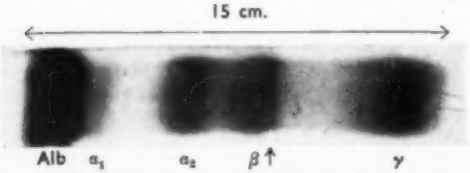

FIG. 2.—The serum protein pattern obtained after electrophoresis in agar gel at pH 8.6.

protein separation. Particularly good separation between the α_1 and α_2 globulins and between the β and γ globulins was obtained. When 0.1 ml. of serum was used, there was very little visible protein trailing between the various protein bands. The deeply stained α_2 globulin region appears to be partly due to lipoproteins in that region (Pezold, 1958).

Staining and Drying

Dye Solution.—To 0.10 g. bromophenol blue and 50.0 g. $ZnSO_4.7H_2O$ add about 25 ml. of 95% ethanol. Mix thoroughly and add 5% (v/v) acetic acid to make 1 litre.

Wash Solution A.—Acetic acid, 5% v/v.

Wash Solution B.—Glycerine, 10 ml., 5 ml. acetic acid, 1 g. sodium acetate, water to 100 ml.

After electrophoresis the gel is placed in the dye solution for eight hours, or overnight, and then washed in solution A until the background is clear. It is finally rinsed in water. After this, the gel is placed on a perspex tray, covered with filter paper, and dried overnight at 37° C. The agar film is then placed for 10 minutes in solution B to enhance the colour of the stained proteins and to make the film pliable. If the agar film has dried on to the filter paper, the filter paper can be removed at this stage. The film is finally hung up to dry at room temperature.

Discussion

Agar gel electrophoresis has many applications in the analysis of serum constituents and is a useful method to have available in the laboratory when required. The technique described enables a simple paper electrophoresis box to be used at any time with agar gel, the only addition necessary being a suitable bridge between the buffer solution compartments. By resting the gel on a celluloid support the necessity for a separate connexion between the gel and buffer solution was eliminated. The buffer and staining solutions were those also employed for paper electrophoresis. The separation of serum proteins was satisfactory and would enable further analysis of the fractions to be carried out. One advantage over the use of paper was that larger quantities of serum could be handled and good separation still obtained. Preparing the gel strips in batches greatly reduced the time taken to set up the apparatus for electrophoresis in agar gel. The time taken to complete the separation and staining of the proteins was not found to be a disadvantage, as the various processes could be left unattended. The changing of the staining and washing solutions took very little time.

Summary

An adaptation of a paper electrophoresis box for use with agar gel and of its use in the electrophoresis of serum proteins is described.

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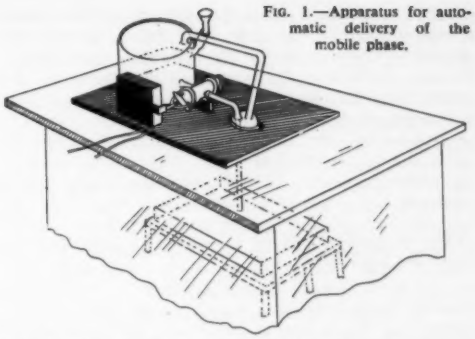

FIG. 1.—Apparatus for automatic delivery of the mobile phase.

Automatic Application of Mobile Phase for Descending Paper Chromatography

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When volatile solvents are used for paper chromatography, as in the systems described by Bush (1952) for separation of steroids, it is usually necessary to hang the paper in the tank for several hours' equilibration before the addition of the mobile phase. If equilibration is allowed to proceed overnight much of the next day will be taken up with running the chromatogram. An apparatus has therefore been constructed which delivers the mobile phase into the upper trough at a pre-arranged time during the night, so that development of the chromatogram is completed by morning.

The mobile phase is contained in a cylindrical glass chamber (Fig. 1) constructed from a 250 ml. beaker or suitable length of wide-bore tubing. The chamber rests on the glass plate forming the roof of the chromatography tank, and is provided with (1) a filling tube, normally closed by a ground-glass stopper, and (2) a horizontal outlet tube, inserted a little above the floor of the chamber, so that any stationary phase which may have been added will not be carried over into the trough. This tube contains a 4 mm. two-way spring-loaded stopcock. Beyond the stopcock the tube turns downwards and passes through the roof of a small glass dome covering a hole in the glass plate, and thence into the upper trough of the chromatography tank. The base of the dome is ground to form an airtight seal with the glass plate.

The handle of the tap is embraced by two curved metal arms, soldered to the spindle of a solenoid-actuated rotary switch. The spindle rotates through 60° when the solenoid is energized. The switch is connected via a "sangamo" 24-hour mains-operated time clock (with separate motor connexions and a day-omitting device) to a 12-volt car battery (Fig. 2). By careful adjustment of the contact arms of the time clock the "on" time can be reduced to 50–70 seconds. As an additional precaution, and to prevent the solenoid heating up unduly, a 12-volt "flasher" (Bulgin thermal delay switch Type S586) is incorporated in the circuit to disconnect the solenoid about seven seconds after the current has been turned on by the time clock. Flasher, battery, and time clock are situated some distance from the apparatus, which can therefore be placed in position inside a thermostatically controlled heating box without danger of sparking in the inflammable atmosphere of the volatile solvents.

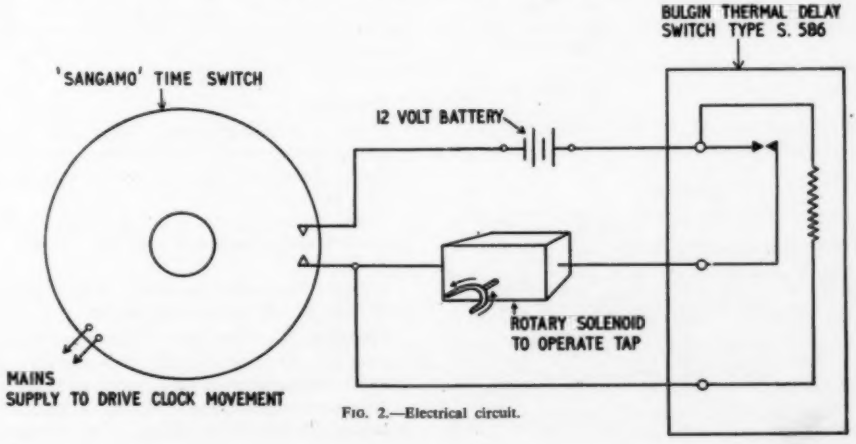
BULGIN THERMAL DELAY SWITCH TYPE S. 586
'SANGAMO' TIME SWITCH
12 VOLT BATTERY
ROTARY SOLENOID TO OPERATE TAP
MAINS SUPPLY TO DRIVE CLOCK MOVEMENT

FIG. 2.—Electrical circuit.

(3) A return tube from the glass dome leads back to the top of the chamber. Air displaced from the chromatography tank by the incoming fluid escapes through the hole in the glass plate into the dome and passes via the return tube to the chamber, thus forming a completely closed system.

Added rigidity is afforded by mounting the apparatus on a "tufnol" plate, to which the glass chamber, stopcock, and rotary switch are firmly secured.

We are indebted to the University of London for a grant for apparatus.

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Practical Laboratory Tests for the Identification of *Proteus* Strains

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(RECEIVED FOR PUBLICATION AUGUST 6, 1958)

The rapid identification of *Proteus* strains is of importance since these relatively non-pathogenic micro-organisms are easily confused with other lactose - negative, pathogenic bacteria such as *Salmonellae*.

In many laboratories *Proteus* species are identified by their urease activity, usually by inoculating bacterial cells into a medium containing urea plus an indicator and noting the colour change after four to eight hours (Rustigian and Stuart, 1943). Since urea is decomposed by autoclaving, the medium must be sterilized by Seitz filtration. This complicates the technique and increases the possibility of contamination.

Proom and Woiwod (1951) proposed the use of a method based on the ability of certain *Proteus* species to form volatile amines. The procedure, however, is rather complicated and could hardly be used for routine diagnostic purposes.

More recently Singer and Volcani (1955) have described a simple and rapid method, based on the decomposition of tryptophan by *Proteus* cells to form a compound giving a brown colour with ferric chloride.

The present paper describes two modified techniques, one for detecting urease activity, and the other a simple spot test for the detection of volatile amines. The two modified methods are compared with the tryptophan-ferric chloride test of Singer and Volcani (1955).

Reagents

Procedure A.—The following are required:

Urea Solution.—Dissolve 1.0 g. urea and 0.1 g. phenolphthalein in 10 ml. 96% ethyl alcohol.

Saline Solution.—Make up 8.5 g. sodium chloride in 100 ml. distilled water.

Dilute Hydrochloric Acid Solution.—Add 1 ml. concentrated hydrochloric acid to 100 ml. distilled water.

Sodium Hydroxide Solution.—Add 0.4 g. sodium hydroxide to 100 ml. distilled water.

Procedure B.—The following are required:

2 : 4 Dinitro-fluoro-benzene Reagent (D.N.F.B.).—Add 0.65 ml. dinitro-fluoro-benzene to 50 ml. acetone.

Potassium Hydroxide Solution.—Dissolve 40 g. potassium hydroxide in 100 ml. distilled water.

Technique

Procedure A: Modified Urease Test.—Sodium hydroxide solution is added drop by drop to the urea solution until the indicator becomes pink. Diluted hydrochloric acid is then added till the solution just becomes colourless. Whatman No. 3 filter paper is impregnated with the colourless urea solution, dried at room temperature, and cut into strips (15 × 5 mm.) which are kept in a dry test-tube.

The bacterial strain to be tested is grown on a liquid or a solid medium. If a liquid medium is used the cells are harvested by centrifugation (2,500 r.p.m.) and the supernatant discarded. Bacteria grown on solid media are scraped off with a bacteriological needle and suspended in 0.1 ml. saline solution in a small test-tube (9 × 100 mm.). After immersing the urea-impregnated filter paper strip in the bacterial suspension the test-tube is placed in a 37° C. water-bath and examined after one to two hours. A red coloration of the filter paper strip indicates the presence of urease in the bacterial cell.

Procedure B: Detection of Volatile Amines by D.N.F.B.—The strain to be tested is grown in nutrient broth (Difco) for 16 to 20 hours. Potassium hydroxide solution is now added to make the medium alkaline.

A filter paper previously impregnated with 2:4 dinitro-fluoro-benzene reagent (D.N.F.B.) is placed over the mouth of the test-tube and held down tightly by a rubber band. The tube is then placed in a boiling water-bath for 15 minutes. Care must be taken that the solution in the test-tube does not come into contact with the impregnated filter paper strip. The upper part of the test-tube should be absolutely dry so that only volatile substances reach the filter paper. A yellow coloration of the impregnated filter paper indicates the presence of volatile amines.

If the reaction is doubtful a drop of concentrated hydrochloric acid is placed on the filter paper and if the colour persists the reaction for volatile amines is positive.

Results

Table I shows that out of all the *Enterobacteriaceae* tested only *Proteus* and Providence strains gave positive tryptophan-ferric chloride reactions. This is in agreement with the findings of other authors (Singer and Volcani, 1955; Falkow, 1957; Thibault and Le Minor, 1957).

TABLE I
BIOCHEMICAL ACTIVITIES OF VARIOUS *ENTEROBACTERIACEAE*

Bacteria Tested	Total No. of Strains	No. of Strains					
		Urease		D.N.F.B.		Tryptophan-$FeCl_3$	
		Positive	Negative	Positive	Negative	Positive	Negative
Proteus vulgaris	46	46		46		46	
,, *mirabilis*	10	10		10		10	
,, *morganii*	10	10		10		10	
,, *rettgeri*	16	16		2	14	16	
Providence strains ..	22		22	6	16	22	
Salmonella ..	7		7		7		7
Shigella ..	7		7		7		7
Escherichia coli ..	7		7		7		7

The urease filter paper strip test described was positive with all *Proteus* species, but negative with Providence strains. This modified urease test proved to be useful also for other bacteria, such as *Corynebacterium pseudodiphtheriticum*, which are known to split urea (cf. Breed, Murray, and Hitchens, 1948). Bacteria grown on various solid media like blood agar, MacConkey, or Kligler iron agar medium, were also used. In some cases when growth from alkaline media was used the filter paper strip became pink immediately after its immersion into the bacterial suspension, but this pink colour faded on further incubation if the bacterial cells were unable to hydrolyse urea. It is thus essential to check the results after one to two hours to avoid false positive results.

The modification described has the advantage that a pure culture is not required as contamination will not interfere with the result of the test.

The method for the detection of volatile amines is based on the quantitative D.N.F.B. assay method described elsewhere (Bachrach, Segal, and Rozansky, 1958). The modified method was positive with *Proteus vulgaris*, *mirabilis*, and *morganii*, whereas *Proteus rettgeri* and Providence strains gave variable results as already described by Proom (1955). *Streptococcus faecalis* also gave a positive D.N.F.B. result, but the amine responsible for this reaction is phenylethylamine (Bachrach, Gery, Sterk, and Rozansky, 1958), and not isoamyl and isobutyl amines which are produced by *Proteus* (Ekladius, King, and Sutton, 1957).

Summary

Two simple and rapid methods for the identification of *Proteus* strains are described. One is a modified urease test employing a filter paper strip impregnated with urea and indicator.

The second method is based on the detection of volatile amines by a filter paper impregnated with 2:4 dinitro-fluoro-benzene.

Results are compared with those of the tryptophan-ferric chloride test.

Dr. G. Altman and Dr. D. Merzbach very kindly provided some of the strains used in these experiments.

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A Ball-mill Emulsifier for 3–5 ml. Volumes

G. I. C. INGRAM

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(RECEIVED FOR PUBLICATION DECEMBER 11, 1958)

The present interest shown by workers on problems of coagulation in intractable, waxy phospholipids has prompted the development of an emulsifier to handle small volumes. The device has also been used for preparing even suspensions of inorganic precipitates and of platelet material.

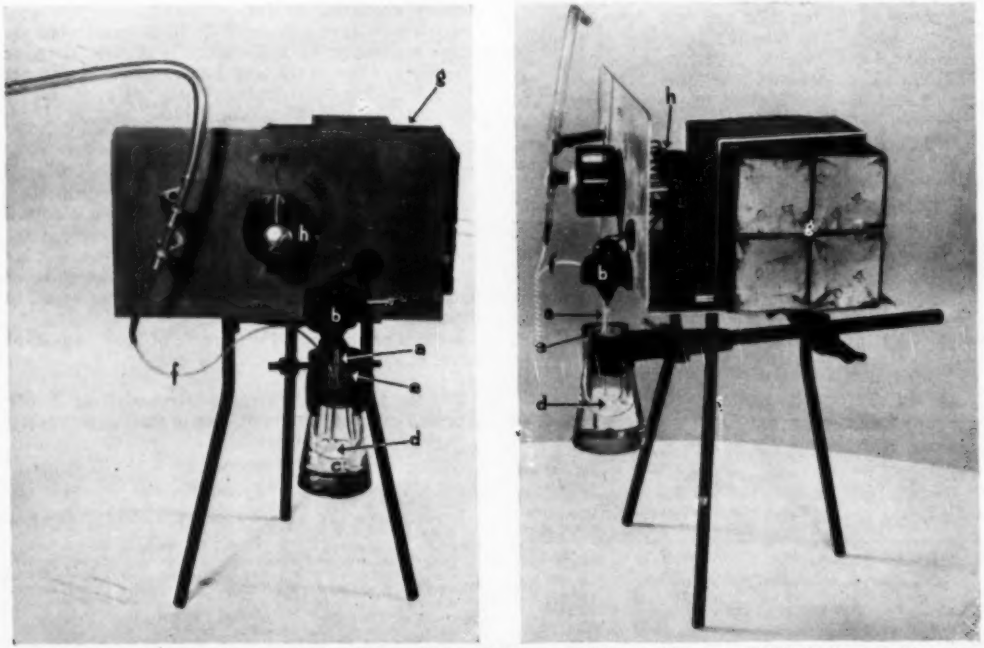
Construction

Principle.—A polythene paddle revolved rapidly by a toy electric motor agitates the liquid in a robust vessel and throws into violent motion a number of glass beads which grind and pound as in a ball-mill.

Method.—The assembly is shown in Figs. 1 and 2. A convenient robust vessel is the lower portion of a Chalk's bottle (which has a rounded cavity, without a corner) held by the neck in a clamp attached to the leg of a tripod. Upon this is mounted the motor, e.g., a 6 v. " mighty midget " without reduction gear, or a 6 v. or 12 v. " micro motor mark III " which may be run at low temperatures (Victory Industries (Surrey) Ltd., Guildford). The tripod also supports the batteries, with as much switching gear as may be thought worth while.

Fig. 3 indicates the relations of the main components. The construction of the paddle and its coupling from Nos. 3 and 4 polythene catheter and copper wire is illustrated in Fig. 4. A somewhat elaborate but convenient electrical circuit is given in Fig. 5.

Convenience is further served by partially closing the neck of the bottle around the shaft of the paddle by fitting a suitably punched polythene bottle closure to prevent splashing, and also by attaching a suction lead, as indicated, to increase the evaporation of organic solvents.



FIGS. 1 and 2.—Front and side views showing the assembled emulsifier. a=Paddle. b=Motor. c=Chalk's bottle, containing glass beads (the appearance of being half-full of liquid is given by the very thick base of the bottle). d=Glass beads. e=Plastic bottle-closure, separately punched for the paddle to pass loosely and for the tip of the suction line to fit tightly. f=Suction line. g=Four bell-batteries. h=Incremental resistance.

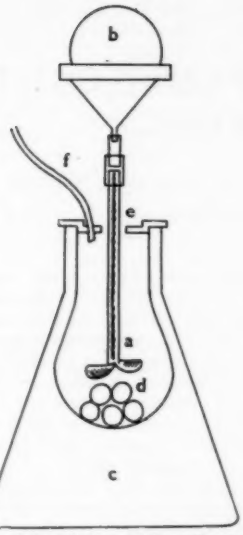


FIG. 3.—Diagram of the relations of the main components. (The key letters are the same as in Figs. 1 and 2.)

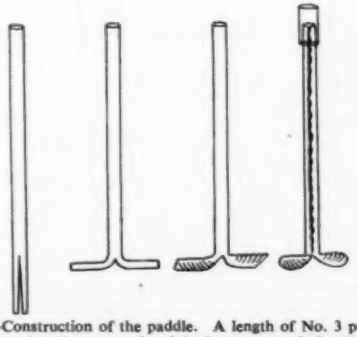


FIG. 4.—Construction of the paddle. A length of No. 3 polythene catheter is split at one end and the flaps separated after immersion in boiling water. The blades of the paddle are moulded with hot forceps and trimmed to the desired size. The shaft is stiffened with wire. The lower end may be sealed with dental cement. The upper end mounts a coupling of No. 4 catheter.

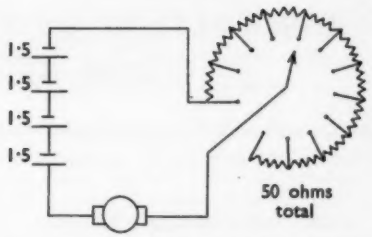


FIG. 5.—A suggested wiring diagram. On the original model, the coil of the incremental speed control resistance was wound by hand, giving 10 positions for the selector switch. A simpler arrangement would be to have four positions representing respectively one, two, three, and four batteries in series.

Use

To prepare a phospholipid "emulsion," pipette the required volume of aqueous vehicle into the Chalk's bottle, e.g., 2 ml. saline. Weigh out the required quantity of phospholipid in a 5 ml. beaker, e.g., 6 mg. Dissolve in ether, say 1 ml. and transfer, with ethereal washings, to the Chalk's bottle. Assemble the emulsifier, apply suction, and start the motor slowly. The ether is evaporated, leaving the phospholipid as a curd in the bottle. Detach suction and increase the speed of the motor until the contents of the bottle are being vigorously churned. Continue until emulsion forms (5–10 min.).

It is a pleasure to acknowledge my indebtedness to a number of colleagues, especially to Dr. D. C. Simpson and Mr. J. Leiper, lately of the Department of Surgery, University of Edinburgh, who respectively suggested the motor and the polythene paddle; to Mr. P. R. Styles and the staff of the Medical Electronics Department of St. Thomas's Hospital, who developed the electric circuit; to Miss P. Leicester, of St. Thomas's Hospital Medical School, for the diagrams; and to the Photographic Department of St. Thomas's Hospital.

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ASSOCIATION OF CLINICAL PATHOLOGISTS: 62nd GENERAL MEETING

The 62nd general meeting was held at the Medical School, University of Manchester, from April 16–18, 1959. Summaries of the papers read there are presented below.

The Myeloproliferative Disorders

H. E. HUTCHISON (Glasgow) reviewed the morbid histology and marrow cytology of the myeloproliferative disorders, beginning with the typical case. Thereafter atypical examples were used to illustrate the relationship to the leukaemias, refractory anaemia both normoblastic and megaloblastic, and reticulum-cell tumour of the haemopoietic system. The value of trephine biopsy and reticulin staining of the marrow was illustrated.

Separation of Red Cell Mixtures by Centrifugation

H. E. M. KAY and M. CONSTANDOULAKIS (Royal Marsden Hospital, London) said that the young red cell, in particular the reticulocyte, is known to have a lower density than the mature cell and can by centrifugation be concentrated in the top layer of the red cell column. Experiments were described which demonstrated the degree of segregation so obtained. It was further shown that, where blood transfusion or bone-marrow transplantation had produced a mixture of circulating erythrocytes, it is usually but not always possible to establish the genotype of the functioning bone marrow by means of such a separation. (See page 312.)

The Principles of the Investigation of Acute Bleeding States Complicating Pregnancy and Childbirth

G. I. C. INGRAM (St. Thomas's Hospital, London) said that the literature describing the acute coagulation disorders which have complicated pregnancy and childbirth was now sufficiently extensive for the principles of the investigation of these disorders to be worked out in detail. Four processes have been thought to cause these defects. They are: (1) Defibrination, which implies a continuing, slow coagulation proceeding in the mother's blood so that the red cells eventually are circulating suspended in serum, with a deposition of the fibrin in various organs. (2) Fibrinolysis, by which is meant an activation of the normal fibrinolytic mechanism of the mother's blood. (3) Release of heparin into the mother's blood. (4) An excessive utilization of clotting factors in an attempt to procure haemostasis at the placental site. In addition, it has been suggested that an insufficient synthesis of clotting factors may contribute in a shocked patient.

These conditions have been seen variously associated with abruptio placentae (concealed antepartum haemorrhage), retained dead foetus, "amniotic infusion," hydatidiform mole, and septic and criminal abortion. The processes of defibrination and fibrinolysis are thought to be initiated by the entry of tissue substances from the conceptus into the maternal circulation, and in these two conditions there have been found thrombocytopenia and reductions in antihaemophilic globulin (A.H.G.), factor V, occasionally factor VII, and fibrinogen.

It is thus apparent that tests might be applied in these cases to detect at least the following abnormalities: thrombocytopenia, heparin - like activity, and depletions of A.H.G., factor V, and fibrinogen. It is important to identify any heparin activity before making other coagulation tests since the presence of heparin will interfere with other techniques. It is suggested that the tests might be arranged in the following order:

(1) *Platelet Count*, and (2) *Thrombin Clotting Time of Citrated Plasma.*—If the thrombin time is longer in the patient's plasma than in the control this might be due either to a reduction in the fibrinogen content or to the presence of heparin. If abnormal, the test should therefore be repeated, adding to both the patient's and the control plasmas first purified fibrinogen and then toluidine blue 1/1,000. This test also provides a method for detecting fibrinolysis, by incubating citrated plasma and repeating the thrombin time on samples withdrawn at intervals over an hour or two; a progressive lengthening of the thrombin time apparent in the patient's plasma but not in the control plasma is strong presumptive evidence of this process.

(3) *Fibrinogen Determination.*—It is probably more useful to estimate fibrinogen as fibrin, i.e., what clots, than in terms of a salt precipitate.

(4) *One-stage Prothrombin Time.*—This will be prolonged where there is a marked diminution in factor V activity. It should be noted in this connexion that in late pregnancy the prothrombin time is shorter than in the non-pregnant state, so that even small prolongations of the patient's time over that of a non-pregnant control should be taken seriously.

(5) *Thromboplastin Generation Screening Test (Hicks and Pitney).*—Using some convenient platelet substitute, this will detect a reduction in A.H.G.

Having made these tests it should be possible to advise the obstetrician whether special infusions, e.g., purified fibrinogen, are required and whether it would be safer to await natural delivery or to intervene under the appropriate cover. The progress of the case may then be followed by repeating those tests shown in the initial investigation to have been abnormal; and in particular, the fibrinogen determination is convenient for following progress since the test is rapid and accurate, the concentration varies fairly rapidly in the plasma following changes in the clinical state or as a result of infusion, and the response to an infusion can be controlled by comparing the expected with the observed increase in the fibrinogen concentration of the plasma.

For these tests (apart from the platelet count) the only requirement is citrated plasma. The reagents for the thrombin–fibrinogen–toluidine blue tests are not difficult to obtain or to prepare and may be stored at −20° C.

It seems advisable to provide the clinicians with a simple screening test such as a rapid fibrinogen determination and to suggest that they call in the clinical pathologist in consultation whenever this test shows abnormal results. The clinical pathologist must therefore provide a service covering 24 hours, but if this can be assured he may be able to offer advice to his obstetric colleagues which is both decisive and life-saving.

A rapid side-room method for estimating fibrinogen was demonstrated, depending upon the increase of turbidity which occurs when plasma clots.

A review of the literature with the full account of the proposed tests is appearing in the *Journal of Obstetrics and Gynaecology of the British Empire.*

Acute Erythraemic Myelosis (Di Guglielmo's Disease)

E. NEUMARK (Department of Pathology, St. Mary's Hospital Medical School, London) said that, since Di Guglielmo's first description of a case of acute erythraemic myelosis in 1926 which followed nine years after he had postulated the existence of such a condition (Di Guglielmo, 1917), more than a hundred cases had been reported in the medical literature. The first case recognized and published in England was Israels's (1939), who called the condition "immature cell erythraemia."

Since 1948 (Britton and Neumark, 1949), when the first case of acute erythraemic myelosis had been shown to the Association, another six cases had been observed at St. Mary's Hospital, but preparations from more than 30 cases had now been seen. Clinically they usually show pyrexia, pallor, and purpura. Most are rapidly fatal.

Haematologically they show anaemia, which occasionally is macrocytic, wide variations of the white blood cell picture ranging from neutropenia to myeloid crises, thrombocytopenia, but occasionally thrombocythaemia, and usually absence of evidence of haemolysis. The marrow shows erythroid hyperplasia sometimes resembling megaloblasts. Pathologically the red bone marrow is increased, but infiltration of other organs is only apparent microscopically.

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Platelet Serotonin and Normal Haemostasis

H. E. HUTCHISON and J. M. STARK (Glasgow). It has often been suggested that the vasoconstrictor, serotonin (5-hydroxytryptamine, 5HT), released from platelets during clotting might take part in normal haemostasis. With the discovery that reserpine can deprive the platelets of this 5HT and prevent further uptake, it has become possible to test haemostasis in the absence of 5HT. No abnormality could be demonstrated when the following investigations were carried out in 12 patients given reserpine: bleeding time, Hess's test, capillary response to injury, silicone clotting time, prothrombin time, prothrombin consumption index, thromboplastin generation test (with platelet dilutions), platelet count, platelet adhesiveness, and plasma clot retraction. From this no evidence was obtained to suggest that 5HT plays any part in normal haemostasis.

Non-healing Granuloma of the Upper Air Passages: Granuloma Gangrenescens

E. W. WALTON (Dundee) said that, though over 300 cases of granuloma which would not heal had now been reported, understanding of it had progressed little since the late Sir Robert Muir wrote in 1897, "The disease does not correspond to any type with which we are familiar." A personal study of 23 cases suggests that "non-healing" granuloma is a tripartite group: classical malignant granuloma, which is a progressive, non-neoplastic but lethal form of ulceration; Wegener's syndrome, which is a disseminated granuloma of hyperergic type originating in the respiratory tract; and granuloma gangrenescens, which is a malignant neoplasm.

Five cases of this third type, having similar clinical and pathological features, were described, and at least 24 others had been reported previously.

Typically, granuloma gangrenescens begins as a prolonged nasal or palatal ulceration: after a latent period of six to 12 months, metastatic lesions develop. Most often, the cervical and mediastinal lymph nodes, liver, skin, kidney, spleen, and lung are affected, with relatively frequent involvement of the heart, brain, small intestine, and gonads. The metastases consist grossly of pale-grey infiltrates and characteristically focal tumours do not occur outside the upper air passages. Histologically these infiltrates are highly cellular and pleomorphic with many mitoses; round and vesicular nuclei are common, and multinucleate cells are quite frequent. The absence of haematological abnormality during life and the absence of atypical cells from vessels in tissue sections support the view that granuloma

gangrenescens is not a leukaemia. There are none of the characteristic features of Hodgkin disease, and most pathologists consider that it is a form of reticulum-cell sarcoma, originating in the nose.

Further Observations Regarding Rheumatic Diseases (Including Rheumatoid Arthritis) and Deficiency of Secretion of the Mucosa of the Small Intestine

ARNOLD RENSHAW (Manchester). A continuance of former work, some of which had been previously reported and which had begun in 1939, stated the results obtained to date by the use of the group of enzymes known as erepsin in the treatment of rheumatic diseases, including rheumatoid arthritis. Some 1,200 patients had now been treated with this enzyme put up in tablet form, the latest preparation being known as "arthrepsin." In addition, at least 10,000 injections of this specially purified enzyme had been given from time to time to these patients in various areas requiring treatment.

The results obtained had on the whole been extremely good. Long-standing and short-standing, severely and slightly affected cases had been included as they presented themselves. In many instances other methods of treatment at present available had been used in various patients without permanent success—cortisone, other medicinals, including large doses of aspirin, sedatives, gold, etc.—before they presented themselves for this treatment. Of 1,200 patients suffering from either rheumatoid arthritis, osteo-arthritis, fibrositis, and various other conditions, such as Paget's disease, spondylitis, Still's disease, 90.9% were improved to some degree and only 9.1% not improved or worse. Of these, 49% were much improved and were practically normal and quite well able to earn a living. Of 533 patients with rheumatoid arthritis, 218 were much improved, 257 were improved, 55 not improved, and only three (0.6%) worse while on treatment and under observation for what is to be regarded as a deficiency disease of intestinal secretion. This latter figure compares with a series of untreated cases of rheumatoid arthritis reported by other observers in which 33% of the patients were worse over five years or so of observation. Improvement occurs in the sedimentation rate, the albumin/globulin ratio, and in the general condition. In patients with osteo-arthritis with hyperpiesia the blood pressure may fall in 12 months or so from 210 mm. to 170 mm. Hg or thereabouts. Patients look five to 10 years younger after a year or so. Associated migraine practically ceases after about three or four months' treatment.

In some cases recovery may appear to be delayed. A full protein diet is required. All purgatives and sedatives should be stopped. Adequate vitamins should be given.

Slides were shown of the condition of the proteins in the swollen bursae when examined by the electron microscope and the effect on these of incubation with erepsin. A cine film also illustrated the improvement in several severe cases.

Cytological Examination of Gastric Washings

A. I. SPRIGGS (Oxford) described the results of cytological examination of gastric washings from 155 patients for malignant cells. A simple saline washing technique was used. Comparative cytological and histological examinations were also made on a series of 24 fresh, surgically excised stomachs.

Of 28 cases with carcinoma, malignant cells were identified in 55% in the first specimen. This figure is lower than many in the literature, but has not been weighted by the results of multiple examinations in cases with strong clinical suspicion, nor by the exclusion of "unsatisfactory specimens." There was one false positive report.

A brief review was given of the techniques used by previous workers in this field, such as abrasive instruments, proteolytic enzymes, and the use of sections of fixed sediment. The last is recommended as a method of relatively low sensitivity, but which requires no special training for technicians or pathologists and takes relatively little time. The examination of smears (which Dr. Spriggs stains either with haematoxylin and mucicarmine or with Papanicolaou's stains) reveals malignant cells in a higher proportion of cases but is extraordinarily laborious.

Coloured slides were shown of some of the principal appearances, mostly in wet-fixed smears but also in sections and in dry films stained by a Romanowsky method.

Some Observations on Drying Hands

K. B. ROGERS (Children's Hospital, Birmingham). The requirements for drying hands in hospitals are different from those demanded anywhere else, and as a busy nurse takes only 10 to 14 seconds to dry her hands any method that is slow is undesirable. The usual method of drying the hands is by employing some form of towel, but it is obvious that a towel will be useless if the act of drying infects the hands. At present nearly all hospital towels are liable to infect the hands.

All the modern hot air hand dryers deliver a current of air that is too narrow to dry a large surface and none dry the hands fast enough for use in hospitals. Moreover, many of the present machines have their air inlets too near the floor, so that infected particles are liable to be sucked up from the floor and discharged into the air.

It must be emphasized that any hospital towel should be covered until it is used, as infected particles fall on it if it is exposed. Paper towels are relatively cheap and provide single disposable towels. Many paper towels are rather rough, tending to scratch the hands, and very few dry the hands when only one is used, so that the time taken to dry the hands is relatively prolonged. Single cloth towels are too expensive to discard and the cost of laundering militates against their use.

Several distributors market towels in long lengths which provide a dry and aesthetically clean surface for drying the hands. The makers are reluctant to alter the design of the machines which deliver the towels, although the present cabinets make it very difficult to use the piece of towel that has been most recently pulled off the unused roll of towel, so that these towels are just as liable to be contaminated as any other exposed towel in a ward in which there is much infection. This has been demonstrated by blowing a small number of very fine particles of wool dust, impregnated with fluorescine, into the air of a room in which such towels are placed. By means of the ultra-violet light from a Wood's lamp it is easy to show how these particles of dust fall on the exposed surfaces.

A cabinet has been designed which will provide a continuous warm, dry, and sterile towel. The cabinet contains boiling water, the steam from which will kill any non-sporing organisms on the surface of a towel, which is drawn through the top of the machine. Experiments showed that there was no damage to the cotton fibres of towels which were placed under tension and exposed to steam continuously for three weeks. The production of this machine is held up because of the cost, as each must have safety devices to prevent over-heating and liberation of steam, and the market would be limited as the demand for these machines would only come from hospitals.

Haemagglutination Reactions with Streptococcal Cultures

F. S. STEWART (Dublin) said that two types of serological change were found in human red cells which had been incubated with streptococcal culture supernatants:

(1) The development of an antigenic modification identical with that occurring in cells modified by exposure to influenza virus (FM1 strain). The agent responsible for this type of change was found in all cultures of pneumococci examined, in a majority of cultures of *viridans* streptococci, in a minority of cultures of streptococci possessing Lancefield group haptens, and was not demonstrable in cultures of *Staph. aureus, neisseriae,* and various Gram-negative organisms, with the exception of one strain of *Salm. typhimurium.* Although not formally proved, the agent responsible for this type of change was believed to be the receptor-destroying enzyme.

(2) Adsorption of heat-stable bacterial antigens probably of polysaccharide nature. Four sensitizing antigens were identified, agglutinins reacting with each being present in most normal human sera. One of these antigens was found to be widely distributed amongst the Gram-positive cocci, being present in most Lancefield group streptococci in all strains of *Staph. aureus* examined and in a minority of cultures of *viridans* streptococci. Except in one case it was not demonstrable in cultures possessing the cell-modifying enzyme. The remaining three antigens were predominantly *viridans* antigens. The possible value of these reactions in streptococcal classification was discussed. A species specific sensitizing antigen was also identified in pneumococcal cultures.

By the use of the sensitization technique it was possible to demonstrate a high titre of antibody in the sera of certain cases of presumed subacute bacterial endocarditis specific for sensitizing antigens in the homologous strain of streptococcus isolated on blood culture and it was suggested that the method might be of value in the diagnosis of this condition.

Benign "Tumours" of the Larynx

S. S. EPSTEIN and I. FRIEDMANN (Department of Pathology, Institute of Laryngology and Otology, London) presented a survey of benign "tumours" of the larynx, based on material seen in their department during the years 1948–58 (see Table). This term was taken to include non-neoplastic and benign neoplastic swellings.

553 BENIGN "TUMOURS" OF LARYNX SEEN AT THE INSTITUTE OF LARYNGOLOGY AND OTOLOGY, LONDON, 1948–1958

A. Non-neoplastic	No.	B. Neoplastic	No.
Vocal cord polyp	428	Papilloma ..	49
Non-specific granuloma vocal process	30	Chondroma ..	2
Intubation granuloma 16		Angioma ..	1
Contact ulcer granuloma 14		Neurilemmoma	1
Primary local amyloidosis ..	5	Lipoma ..	1
Retention cyst	23	Plasmacytoma	1
Adenomatous hyperplasia ..	8		
Granular myo(fibro)blastoma ..	4		
Total	498		55

Vocal cord polyps and their clinical variants, nodes and polypoidal degeneration, are by far the commonest non-neoplastic laryngeal "tumours." They arise as a result of an exudate in the closed subepithelial space of the membraneous cord. Their structure may be fibrovascular or oedematous, and they may, for this reason, be misinterpreted as angiomas, fibromas, or myxomas, which in fact are excessively rare in the larynx. Hyalinization in the vocal cord polyp is common, and when massive must be distinguished from the deposits of primary localized amyloidosis. Non-specific granulomas can occur anywhere in the larynx, but are particularly common on the vocal process, where they may follow the trauma of endotracheal intubation (intubation granuloma), or of forceful apposition of both vocal processes following abnormal patterns of phonation (contact ulcer granuloma).

Cysts and glandular hyperplasia may occur in the larynx. When the glandular structure is conspicuous, compact, and unassociated with a significant inflammatory exudate, they may be regarded as adenomas. Myoblastomas are also rare, and, while their precise aetiological status is doubtful, are of special interest in that the overlying epithelium of the larynx may assume a "pseudo-epitheliomatous" appearance.

Laryngeal papillomas are the commonest benign laryngeal neoplasms and when occurring in childhood tend to be multiple and multicentric in origin. Adult papillomas are more often solitary and localized to the vocal cords and, when recurrent, the possibility of a malignant change must be excluded. Papillary projections from a keratotic epithelium (papillary keratosis) can, clinically and histologically, simulate papillomas, but the conditions are quite distinct. Chondromas are rare and usually arise endolaryngeally from the posterior plate of the cricoid cartilage. Angiomas, neurilemmomas, plasmacytomas are rare.

Pulmonary Bone Marrow Embolism in Accident Reconstruction

J. K. MASON (Royal Air Force) discussed the use of pulmonary tissue embolism as a method for timing injuries. Fat embolism, which had been suggested as being of value, was unsatisfactory because of the frequency with which it occurs and confusion surrounding its genesis. Pulmonary bone marrow embolism is accepted as arising only following trauma to bone and its incidence had been studied in 102 aircraft deaths associated with fracture. Emboli were found in 40.2% of such cases. If a minimum of five slides had been examined in every case, the incidence would have approximated to 60%. Emboli were distributed at random in the lungs.

A correlation with injuries sustained shows that emboli are uncommon when there is evidence of widespread, instantaneous destruction of the organs and, in particular, the cardiovascular system. Emboli can only form in the presence of an intact circulation, but both fat and bone marrow emboli arise with very great speed, the latter being found in 37% of deaths believed to be immediate. Both become more marked with increased survival time, but bone marrow emboli are resolved very much faster than fat emboli. An analysis of the two types of embolism can give valuable information as to survival following injury.

The application of these findings to accident reconstruction was illustrated with reference to deaths following escape from aircraft by ejection. Deaths due to simple lack of height can be differentiated from those associated with injury at altitude by means of a thorough search for pulmonary bone marrow emboli.

The possibility of extending such principles to other types of accident was discussed.

Mesonephric Tumours of the Female Genital Tract

F. A. LANGLEY, LOIS STENT, and T. WADE-EVANS (Manchester) said that Schiller in 1939 described a group of rare tumours characterized by the presence of "glomerulus-like" structures and tubules lined by "hob-nail" cells. He called these tumours mesonephromas because he thought that they were derived from the mesonephros. Teilum (1945) does not accept this view and considers such tumours to be closely related to embryomal carcinomas and chorion-epitheliomas. Teilum himself has described an unrelated class of tumour which he considers to be truly mesonephric.

A study was made of nine tumours belonging to the class described by Teilum. These show much variation in histological pattern. Five main patterns are recognizable, namely, adenomatoid, tubular, cystic, papillary, and sheets, or cords, or eosinophilic cells. Several of these patterns are usually present in any one tumour.

It is concluded that these tumours fall into two groups: (1) Tumours which are truly mesonephric, being derived from vestiges of the mesonephros or from mesenchyme retaining mesonephric potencies; (2) tumours derived from vestiges of the rete ovarii or from mesenchyme retaining rete potencies.
testis.

Tumours similar to these two types occur in the

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Hypernephroma with Polycythaemia: Uraemic Anaemia; Possible States of Over- and Under-secretion of Erythropoetin

H. F. BARNARD (Westwood Hospital, Beverley, Yorks) said that the association of these states had, in recent years, been reported a number of times. The case here described, a woman of 73, suffered from haematuria, a haemoglobin of 135% repeatedly, and no other notable feature. A moderate-sized typical hypernephroma was removed by nephrectomy. Histologically the tumour was a typical clear cell type with no distinguishing features. The haemoglobin returned to normal and the patient remains well with a normal haemoglobin a year later.

Polycythaemia occurs with probably 4% of hypernephromas and may be the presenting symptom. Erythropoetin long suspected and now reasonably established is probably secreted by the kidney. It has been suggested that in polycythaemic cases the tumour may secrete erythropoetin.

In uraemic anaemia it is reported that erythropoetin in the plasma is low. It is of interest, therefore, that by transfusing post-haemorrhagic plasma into uraemic anaemia reticulocytosis has been obtained.

Birth Injury to the Neck

P. O. YATES (Department of Pathology, University of Manchester) said that all methods of delivery involved stresses of varying degree to the infant's neck. Out of 213 deaths occurring in the perinatal period, a random selection of 60 were examined histologically for evidence of trauma to certain cervical structures. Though only two, both breech deliveries, showed marked bruising of the spinal cord, nine had nerve root injuries, and in nearly half the total there was damage to the spine ligaments and the dura were torn in some.

The most surprising findings were haematomas in the walls of the vertebral arteries in 24 cases. Many

of these were large enough to cause narrowing of the arterial lumen. They were thought to arise from tearing the origins of arterial twigs supplying nerve roots and spinal cord. These haematomas may be of considerable importance since ischaemic damage to the nuclei of the brain-stem would result in cardiorespiratory embarrassment and dysfunction of cranial nerves. Moreover, since the vertebral arteries are responsible for the blood supply to the inner ear, the optic cortex, and in part the basal ganglia, those infants surviving moderate degrees of ischaemia might later show deafness, blindness, or one of the forms of cerebral palsy. The bilateral symmetry of some cerebral lesions described in cerebral palsy which has in the past been attributed to interruption of venous drainage into the great vein of Galen could equally well be accounted for by stenosis of a vertebral artery, since these vessels have, via the basilar artery, a bilaterally symmetrical distribution.

Serum Isocitric Dehydrogenase in the Investigation of Liver Cell Damage

D. N. BARON and JOYCE L. BELL (Royal Free Hospital, London) reported that serum isocitric dehydrogenase (ICD) was estimated by the method of Wolfson and Williams-Ashman (1957), involving spectrophotometric measurement at 340 mμ of conversion of T.P.N. to T.P.N.H. during dehydrogenation and decarboxylation of D-isocitrate. Activity is measured in units similar to transaminase units: one unit is a change in O.D. of 0.001/ml. serum/min. in a volume of 1.5 ml., corrected to 25° C. (Q_{10}; 20°–30° C.=1.7). Our normal range was 2.5–9 units; that of Wolfson and Williams-Ashman, converted to these units, was 3–15 units (1 O.D. unit=15 Wolfson reactant units).

Hepatocellular damage raised the serum I.C.D. High values (up to 200 units) occurred in parallel with transaminases in infective hepatitis, and a rise (20–60 units) was found following administration of hepatotoxic drugs, e.g. iproniazid, in primary and metastatic hepatic malignant disease, and usually in cirrhosis. Serum I.C.D. was consistently normal in post-hepatic obstructive jaundice, and this may prove valuable in differential diagnosis.

In clinical protein malnutrition (in Nigeria) serum I.C.D. rose (average 30 units), and slight increases (average 10 units) were found in villagers on a poor diet. Serum I.C.D. appeared more sensitive for liver damage here than all other tests, including transaminases.

In uncomplicated myocardial infarction, serum I.C.D. was normal, but in cases with heart failure and liver enlargement moderate rises were found; in one case with prolonged hypotension, the serum I.C.D. exceeded 200 units.

REFERENCE

Wolfson, S. K., and Williams-Ashman, H. G. (1957). *Proc. Soc. exp. Biol. (N.Y.)*, **96**, 231.

The Diagnostic Value of Plasma Amylase with Special Reference to Gastrectomy

G. K. MCGOWAN (Bristol) said that over a seven-year period 53 patients who might have been diagnosed clinically as acute pancreatitis were found to have plasma amylase levels of over 1,000 Somogyi units/100 ml. In 39 the diagnosis was certainly or very probably acute pancreatitis, and in five the final diagnosis was uncertain. In nine the diagnosis was shown not to be acute pancreatitis.

Of these nine "false positives," seven had had a partial or total gastrectomy at a time which varied from 12 hours to four years before the attack, whereas none of the true cases of acute pancreatitis had had a gastrectomy. The two remaining "false positives" were suffering from perforation of a peptic ulcer.

A further four patients on whom a gastrectomy had been performed developed abdominal symptoms with plasma amylase levels of over 1,000 units, but none of these were considered on clinical grounds to be suffering from acute pancreatitis.

After gastrectomy there are many possible factors which can contribute to a rise in the plasma amylase level such as resistance to the escape of pancreatic secretions due to afferent loop obstruction or spasm of the sphincter after drugs like morphia; absorption of amylase from the peritoneal cavity after escape of pancreatic secretions due to leak from the gut or accidental secretion of accessory pancreatic ducts; secondary pancreatitis resulting from peritonitis; renal insufficiency in post-operative shock reducing the excretion of amylase in the urine.

It was concluded that a high plasma amylase should not be taken as indicative of acute pancreatitis if the patient has had a gastrectomy, and that pathologists should emphasize this point when reporting such raised values.

Serum Transaminase Activity in Cirrhosis of the Liver and Related Conditions

K. O'SULLIVAN and J. KOHN (Roehampton) had classified the 450 patients who comprised this study as follows:

Liver diseases	230 (114 cirrhosis)
Miscellaneous diseases	155
Patients with or suspected of cardiac infarction	35
Normal subjects	30

The S.G.O.T. and S.G.P.T. activity was observed in a large number of cases of chronic liver disease and the results compared with the flocculation liver function tests, the alkaline phosphatase, serum proteins, E.S.R.s, and electrophoresis of serum proteins.

No direct correlation was found between these tests and the serum transaminases. The conclusion was reached that the serum transaminases are far more sensitive tests than the flocculation tests in the assessment of hepatocellular damage, and that abnormal serum transaminase values quickly follow hepatic necrosis. In acute cases the rise in serum

transaminase may precede abnormal readings of all the other liver function tests.

Alkaline phosphatase was estimated in 300 cases in conjunction with the serum transaminases and in 26 liver cases abnormal alkaline phosphatase levels were found. Of these 26, 80% also had abnormal serum transaminases. On the other hand, 68 liver cases with abnormal serum transaminases were found; of these only 31% had abnormal alkaline phosphatases. A greater percentage of cases of cirrhosis with a previous history of infective hepatitis had abnormal serum transaminase values than other cases of cirrhosis. The incidence of inverted S.G.O.T.–S.G.P.T. ratios in chronic hepatic diseases was noted. A reversed ratio was present in 40% of liver cases with abnormal serum transaminases and in 19% of liver cases with normal transaminases.

When the serum transaminases are estimated by the colorimetric method of Reitman and Frankel (1957) corrections for temperature are unnecessary in the range of 20 to 30° C.

It is concluded that in chronic liver diseases the serum transaminases are a valuable adjunct in assessing clinical progress and as a guide to prognosis.

REFERENCE

Reitman, S., and Frankel, S. (1957). *Amer. J. clin. Path.*, **28**, 56.

The Effect of Heparin on the Early Stages of Blood Coagulation

J. R. O'BRIEN (Portsmouth). Heparin in critical dosage was found to have no apparent effect on anti-haemophilic globulin, factor V, factor VII, or on platelet activity, and the activation of coagulation by glass (Hageman factor) was not affected. Evidence was presented which strongly suggests that heparin actually combines with and inactivates Christmas factor and that the addition of protamine sulphate to such a heparin-Christmas factor complex removes the heparin, thereby liberating the Christmas factor activity. Heparin can apparently exist in two forms in plasma. It may be free and available to delay clotting, or it may be inactivated, presumably bound to some protein, and unavailable to delay clotting. The ability of coagulation factors to inactivate heparin was investigated; the clotting time of a thrombin fibrinogen mixture was used to measure the presence or absence of available heparin after the heparin had been added to the plasma fraction under study. Plasma Christmas factor, and especially serum Christmas factor, and platelet protein, but not intact platelets, were shown to inactivate heparin. The platelet-like activity of serum (O'Brien, 1955) also inactivated heparin. The ability of whole native blood to inactivate heparin was monitored while the blood was clotting and the amount of heparin that could be inactivated was markedly increased in the subsamples taken soon after the blood started to clot; this increased inactivating power of blood was due presumably to platelets breaking up, and to the formation of platelet-like activity of serum and serum Christmas factor, and these three occurrences probably accounted for all the heparin inactivated. Critical quantities of heparin added to whole native blood delayed clotting, probably due to its effect on Christmas factor. Thrombin generation and prothrombin consumption were normal and no detectable heparin remained in the serum; presumably the heparin was inactivated by the mechanisms reported above before it could interfere with the intrinsic thrombin formed.

REFERENCE

O'Brien, J. R. (1955). *Brit. J. Haemat.*, **1**, 223.

Problems in the Diagnosis of Megakaryocytic Myelosis

H. G. H. RICHARDS (Winchester). The main clinical and laboratory findings in myelosclerosis were reviewed, and the three stages of this disease described by Leonard, Israels, and Wilkinson (1957). A series of five cases of megakaryocytic myelosis which appeared to be a special form of myelosclerosis, and might be related to stage I, as described by Leonard *et al.*, were described.

Clinically, these cases featured refractory anaemia with hepatosplenomegaly and wasting. Laboratory investigations revealed a progressive anaemia with marked erythropoikilocytosis, bizarre platelets in circulation, and a leucoerythroblastic blood picture. Marrow aspiration usually produced blood only. At necropsy (or by means of a surgical biopsy of the marrow) it was found that the marrow was hypercellular, with a superabundance of megakaryocytes, and an increase in reticulin stroma. The liver sinusoids showed foci of haemopoiesis, including megakaryocytes, and the spleens, in available sections, likewise showed the presence of megakaryocytes with other evidence of myeloid ectopia.

The differential diagnosis between megakaryocytic myelosis and other conditions such as myeloid leukaemia, a necrotizing form of Hodgkin's disease, typical myelosclerosis, and leucoerythroblastic anaemia arising in secondary carcinomatosis of bone marrow, was illustrated by examples.

Hypofibrinogenaemia in Pregnancy: Prevalence and Prognosis

R. F. JENNISON (St. Mary's Hospitals, Manchester). A survey of the incidence of hypofibrinogenaemia in pregnancy was carried out at St. Mary's Hospital, Manchester, during a period of a little more than three years, the object being to assess the frequency and severity of this condition.

One hundred and ninety fibrinogen estimations were carried out at all stages of normal pregnancy. The mean fibrinogen level in the first trimester was 377 mg.%, in the second trimester 443 mg.%, and in the third trimester 487 mg.%. The standard deviation in the third trimester was ±102 mg.%, so that the limits within which 95% of fibrinogen levels in normal pregnancy should fall were 283 mg.% to 691 mg.%, and, on this basis, when reviewing the

patients in the survey, fibrinogen levels below 270 mg.% were considered possibly to be due to some pathological condition.

In the survey, fibrinogen estimations were done on (1) patients with an accidental haemorrhage, (2) women with retention of a dead foetus *in utero* for longer than one week, (3) patients with severe shock or with haemorrhage due to causes other than accidental haemorrhage and placenta praevia, (4) patients with severe toxaemia of pregnancy or eclampsia. Two hundred and six women with these conditions were investigated and 52 were found to have hypofibrinogenaemia.

Thirty-four women with low fibrinogen levels were found in 117 patients with accidental haemorrhage. In 1956, 81 patients, in 1957, 100 patients, and in 1958, 82 patients were admitted with accidental haemorrhage according to the hospital records. The incidence of hypofibrinogenaemia in those years was 9%, 13%, and 12% of the patients with an accidental haemorrhage. Of the 34 patients with low fibrinogen levels, eight were below 50 mg.%, six between 50 mg.% and 100 mg.%, and 10 between 100 mg.% and 200 mg.% The more severe deficiencies in fibrinogen usually occurred in the patients with large retroplacental blood clots. All but three of these patients had transfusions of blood and/or plasma. The treatment was usually started because of shock or haemorrhage and not because of the low fibrinogen level. Of the 10 patients with fibrinogen levels above 200 mg.%, only three had transfusions. The fibrinogen levels returned rapidly to normal in all cases irrespective of treatment.

Fifty-three women with a dead foetus retained *in utero* were also investigated. Forty-one of these retained the dead foetus for three weeks or more and 13 of these developed hypofibrinogenaemia. In one patient the fibrinogen level fell below 50 mg.%, in two it was between 50 mg.% and 100 mg.%, and in six between 100 mg.% and 200 mg.%. There was a progressive slow fall in all cases, the lowest levels being reached in those women who retained the dead foetus the longest. Only three of these patients had transfusions and the fibrinogen levels returned rapidly to normal. The only severe haemorrhage was in a patient delivered immediately on admission to hospital.

Amongst the miscellaneous conditions were two patients with haemorrhage associated with abortion and fibrinogen levels of 68 mg.% and 22 mg.%. One other patient was admitted with severe haemorrhage and a ruptured uterus. Her fibrinogen level on admission was 150 mg.%. Two other patients became extremely shocked early in labour and a diagnosis of amniotic fluid embolism was made. The fibrinogen level fell to 50 mg.% in the first patient. No fibrinogen was detected in the second patient until seven hours after delivery. Multiple transfusions were required, but both patients recovered so that the diagnosis of amniotic fluid embolism could not be confirmed by necropsy.

No instances of hypofibrinogenaemia were seen in toxaemia of pregnancy.

Fibrinogen was only used in the treatment of these patients on one occasion. When treatment other than blood transfusion was thought to be necessary, triple strength plasma was used.

All the patients, except one who had a caesarean section for suspected placenta praevia, were delivered vaginally.

In all the patients delivered in hospital a prior knowledge of the fibrinogen level has allowed extreme care to be taken in order to avoid unnecessary trauma and following delivery the uterus has been kept well under control.

The patients who caused most concern were those delivered either outside the hospital or without knowledge of the fibrinogen level, or those in whom some abnormal condition made the obstetric management difficult.

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BOOK REVIEWS

Neuropathology. By J. G. Greenfield, W. Blackwood, A. Meyer, W. H. McMenemey, and R. M. Norman. (Pp. vi+640; 369 figures. 105s.) London: Edward Arnold. 1958.

This may be regarded as a monument to the doyen of British neuropathologists, Dr. Godwin Greenfield. The book was planned and edited by Dr. Greenfield, and not only was at least half of it written by him, but he advised on the sections written by his collaborators. Dr. Greenfield's knowledge of the pathology of the nervous system, studied by the classical methods of histopathology, was unrivalled, and in those sections of the book written by him will be found his matured views on a subject of which he was a master. The chapters on the general pathology of the cell and neuroglia, infections, traumatic and demyelinating diseases, as well as those on system degenerations, neuritis, and neuropathy, and lesions of the nervous system associated with malformations of the skeleton are all contributed by Dr. Greenfield himself.

Professor Meyer draws on his wide and unique experience to contribute valuable sections on epilepsy, psychoses of obscure pathology, and the effect of anoxia, intoxications, and metabolic disorders on the nervous system. This is an erudite section by one whose knowledge of the literature of these subjects is unsurpassed. Dr. Norman writes on malformations of the nervous system, birth injury, and diseases of early life, and has produced a beautifully illustrated chapter, the illustrations being from his own truly remarkable collection of cases. He is to be congratulated on the way in which he has presented an extremely difficult subject on which our knowledge of the fundamental disease processes is as yet woefully lacking. Another subject in which the pathology is obscure and the literature difficult and often conflicting is that of the dementias and progressive diseases of the basal ganglia, but Dr. McMenemey has presented a useful synthesis of what is known of the pathology of these diseases. Professor Blackwood contributes a most informative and valuable chapter on vascular disease of the central nervous system, and since cerebral vascular disease is one of the major causes of damage to and degeneration of the brain the importance of this subject cannot be overestimated.

As is inevitable with books of multiple authorship this volume shows a considerable degree of patchiness, and in places the emphasis is extremely odd. For instance, syphilis of the nervous system takes up 17 pages, while tuberculosis has only six devoted to it and poliomyelitis but four. The section on trauma of the nervous system needs extensive revision.

The bibliography is fairly complete, though there are surprising omissions, and it would be much improved if the titles of the articles to which it refers were given, as is done in the better books and journals. For a textbook which sets out to be a serious work of reference, the absence of titles in the bibliography is, in this reviewer's opinion, a black mark. It is a cheeseparing and unworthy economy. There is also an annoying number of mistakes which will have to be corrected in the next edition, when this is called for, as it certainly will be. The index, so important a feature of a (good) work of reference, leaves much to be desired.

Nevertheless, this book, with its companion volume on the pathology of cerebral tumours, to be published in the near future, will for long remain a basic text on neuropathology. There is no great work on the pathology of the nervous system in the English language comparable with the gigantic German monograph edited by Henke and Lubarsch in five fat volumes, and Greenfield's book is the most complete text that is available to the English-speaking world. Even though all the available information is not always to be found, the inquirer can usually trace the original sources.

The book will be an essential reference work for the private libraries of all pathologists, and the less they have to do with the nervous system the more essential will the book be, for it will help them as can no other when a problem on the nervous system turns up in their work.

It will keep alive the memory of Godwin Greenfield, which will long be warmly remembered by pathologists.

P. M. DANIEL.

The Borderland of Embryology and Pathology. By R. A. Willis. (Pp. ix+627+index; 244 figures. 90s.) London: Butterworths. 1958.

Most practising pathologists have at some time felt the need for a greater knowledge of embryology to assist them in solving problems connected with the diagnosis of malformations. To those who have been fascinated by reading chapters in Bland-Sutton's book on tumours and Gould and Pyle's *Anomalies and Curiosities of Medicine*, Professor Willis's more basic and complete work will be especially welcome.

Although the material in the introductory chapters is available in textbooks of embryology, it is essential to have a clear and concise account of normal development as a prelude to the more practical information which is to follow. In the main part of the work where the emphasis is of necessity on structure it is pleasing to see functional aspects also treated, as, for example, in the discussion on the possible existence of cortical adrenal hormones in the foetus. Later on, when hamartomas are being strictly defined, the genetic aspects of these malformations are compared with those of inborn errors of metabolism, thus emphasizing once more the

necessity of allying structure with function. It is particularly gratifying to find a subject such as metaplasia, which receives scant attention in many works on pathology, dealt with so completely. When discussing repair and regeneration some clarity of definition is lost and it is a pity that a confusing and contradictory term such as "compensatory regenerative hyperplasia" has found its way into an otherwise excellent chapter. At the present time when the wider aspects of immunity are becoming appreciated the chapter on transplantation will be enjoyed by all.

It must have been difficult choosing a title for a work which deals with so many different topics. As in previous works Professor Willis has wisely selected essentially practical problems which concern pathologists and ones on which reliable information cannot always be readily obtained. The clarity of exposition and the concise rendering of the facts are well-known characteristics of the writer. Lists of references are very complete and it is again most valuable for the busy pathologist to have important review articles indicated for him in heavy type. Professor Willis is to be warmly congratulated on his latest work, and it is hard to see how any pathologist alive to his daily problems will be able to avoid the necessity of having a copy on his shelves.

GEORGE CUNNINGHAM.

Le Cancer de la Corde Vocale. By J. Piquet. (Pp. 204; 53 figures. Fr. fr. 1,600.) Paris: Masson et Cie. 1958.

This monograph on cancer of the vocal cord by Professor Piquet, of Lille, is the latest of an international series on otorhinolaryngological subjects.

The monograph contains chapters on aetiology, pre-cancerous states, pathology, and treatment. The style is somewhat discursive, and references are haphazard and inadequate. In a sketchy chapter on laryngeal and cervical lymphatics, in which just one poor and unlabelled illustration appears, there is the following reference:

> "Pour la description classique des lymphatiques du larynx, le lecteur pourra se reporter a l'excellent *Traité d'anatomie de Rouvière*, où la quesion est traitée dans tous ses détails." (!)

Other references are to obscure sources or simply short quotations from discussions at meetings. A bias towards the Continental literature has occasionally resulted in serious lacunae. For instance, the author claims that teleradium techniques are of little value in the treatment of cancer of the larynx without even mentioning the important contributions and results of Lederman in this country. It is furthermore difficult for the English reader to accept Piquet's recommendations for local anaesthesia in total laryngectomy, but it must be remembered that this is in keeping with still much of Continental practice.

This monograph contains much material of interest. It is only a pity that it is marred by careless references and parochialism.

S. S. EPSTEIN.

Radioisotope Laboratory Techniques. By R. A. Faires and B. H. Parks. (Pp. xii+244; illustrated. 25s.) London: George Newnes. 1957.

The increasing use of radioactive isotopes in general laboratories has made necessary a simple book providing the essential theory and practice of radioisotope techniques. The authors, with considerable teaching experience of the basic principles at the Harwell isotope school, have attempted to produce such a book, primarily for those with only an elementary knowledge of physics. Consequently, they have been compelled to be selective and have concentrated on practical details of choosing and using equipment, theory being kept to a minimum. Suggestions for further reading are given at the end of most chapters.

The first few chapters deal concisely and clearly with the elements of nuclear physics, the properties of radiations, the production of radio-isotopes, and the calculation of radiation dose rates. The chapters which follow, describing laboratory design, radioactive hazards, decontamination, and laboratory apparatus, contain much useful data and practical advice. The major part of the book, dealing with instrumentation and applications, is, however, not quite so successful. The reader with no knowledge of electronics, for whom this book is intended, would soon get lost in the technicalities of the electronic apparatus described. The authors have condensed the chapter on electronic techniques to the point where the explanation of terms frequently used in later chapters is either omitted or incomprehensible without previous knowledge.

Several chapters are devoted to descriptions of the construction and use of particle and radiation detecting equipment. These chapters are uneven in quality. As an example, the authors describe in great detail the types of gases used in Geiger-Müller counters, but give only a sketchy account of the use of some of these counters with no account at all of the use of liquid counters. Scintillation counters are dealt with inadequately, and the chapter on the choice of counting equipment is too brief. On the other hand, the important subject of the statistics of counting is adequately treated by rule-of-thumb methods.

The final chapters describe briefly some chemical and other applications of radio-isotopes and autoradiography (in rather more detail). No account is given of the use of radioactive tracers in medical or biological work. Although the ideal book for non-technical users of radioisotope techniques remains to be written, this book may be recommended as a useful practical guide to the subject.

M. LUBRAN.

Textbook of Virology, 3rd ed. By A. J. Rhodes and C. E. van Rooyen. (Pp. xv+607; 81 figures. 80s.) London: Baillière, Tindall & Cox. 1958.

This edition, larger by 70 pages, maintains the high standard of presentation of the 1953 edition. It is beautifully printed and well illustrated. The authors'

lucid exposition makes for pleasurable reading and they attain their declared objective of presenting an account of the essential features of the virus and rickettsial diseases of man.

The book is designed to appeal to a wide variety of readers, but in order to assist those with a clinical orientation, the virus infections of man are described "depending on the system primarily affected" (p. 5). Since human infection by many different viruses can give rise to the same syndrome, e.g., aseptic meningitis, and because infection with a single virus may cause in man such a diversity of pathological signs as to confound the choice of a system primarily affected, e.g., mumps, this arrangement of the text introduces difficulties. The authors have been at pains to try to overcome these, but the result is not wholly satisfactory from either the clinical or virological viewpoint.

A fundamental drawback is the spatial separation of viruses, which, because they have properties in common, may be considered to form a group. Thus the agents of psittacosis, lymphogranuloma venereum, and trachoma are described in separate, albeit consecutive, sections. Diseases due to Coxsackie viruses form a section on their own, whereas poliomyelitis and poliomyelitis-like illnesses (Icelandic disease and "ECHO" viruses) appear in the section entitled neurotropic virus diseases in company with rabies and the arthropod-borne encephalitides. Other arbor viruses are in another section on arthropod-borne and tropical fevers.

However, these are criticisms of arrangement and not content. Cross-reference is made easy by indications in both the text and index.

Of particular appeal to general practitioners, pathologists, and students alike will be the references for further reading, and in particular the annotations on laboratory diagnosis. The ever-growing demand for such information and its practical importance in getting the right specimens, at the right time, in a suitable condition for laboratory diagnosis, cannot be over-emphasized.

It is a pleasure to recommend this textbook to everyone requiring a handy reference book on the subject.

G. P. B. BOISSARD.

Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Mr. G. S. Corden, Swan Press Ltd., 20 Bakers Row, Farringdon Road, London, E.C.1,** price 1s. each.

No.	Title	Date	Author
1	The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics	1952	R. W. Fairbrother
2	Determination of Sensitivity of *M. tuberculosis* to Streptomycin	1952	R. L. Vollum
3	The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents	1953	L. C. Nickolls
4	The Estimation of Carbon Monoxide in Blood	1953	D. A. Stanley
5	The Identification of Reducing Substances in Urine by Partition Chromatography on Paper	1953	G. B. Manning
6	The Paul-Bunnell Test	1954	R. H. A. Swain
7	The Papanicolaou Technique for the Detection of Malignant Cells in Sputum	1955	F. Hampson
8	Investigation of Haemorrhagic States with Special Reference to Defects of Coagulation of the Blood	1955	E. K. Blackburn
9	Daily Fat Balance	1956	A. C. Frazer
10	Mycological Techniques: (1) Collection of Specimens	1956	R. W. Riddell
11	Mycological Techniques: (2) Cultural Isolation	1956	R. W. Riddell
12	Techniques for Demonstrating L.E. Cells	1956	J. V. Dacie and L. S. Sacker
13	The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis	1956	Joan Taylor
14	The Determination of Serum Iron and Serum Unsaturated Iron-binding Capacity	1956	Arthur Jordan
15	The Estimation of Faecal "Urobilinogen"	1957	C. H. Gray
16	Preservation of Pathological Museum Specimens	1957	L. W. Proger
17	Cultural Diagnosis of Whooping-cough	1957	B. W. Lacey
18	The Rose-Waaler Test	1957	C. L. Greenbury
19	The Laboratory Diagnosis of Fibrinogen Deficiency	1958	R. M. Hardisty
20	Investigation of Porphyrin	1958	C. Rimington
21	Quantitative Determination of Porphobilinogen and Porphyrins in Urine and Faeces	1958	C. Rimington
22	Investigation of Haemolytic Anaemia	1959	J. G. Selwyn
23	The Dried Disc Technique for Bacterial Sensitivity Tests	1959	R. W. Fairbrother and J. C. Sherris

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BRAIN CHANGES IN RUPTURED INTRACRANIAL ANEURYSM

BY

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Since the papers of Beadles (1907), Fearnsides (1916), and Symonds (1924), there have been numerous publications on the clinical manifestations, prognosis, and treatment of spontaneous subarachnoid haemorrhage. The pathology of the brain has received less attention. Many accounts have been taken from routine necropsy reports and few have included detailed examinations of the brain. Intracerebral bleeding has received most attention, largely since the paper of Richardson and Hyland (1941), but recently ischaemic lesions have been more widely recognized (Robertson, 1949; Wilson, Riggs, and Rupp, 1954; Bebin and Currier, 1957). Robertson (1949) described five instances of ischaemia, and it is noteworthy that three were seen in the 10 cases he observed himself, whereas only two were recorded in more than 80 routine post-mortem reports he reviewed. The greatest incidence of infarction is reported by Wilson *et al.* (1954), who noted focal ischaemic lesions in 45% of their necropsy material. No account, to my knowledge, however, gives details of the distribution, size, and importance of ischaemic lesions and their relation to other findings in the brain.

This paper reports the brain findings in a series of 32 consecutive cases of fatal ruptured intracranial aneurysms. Two further instances have been excluded because surgical intervention made assessment of the pre-operative findings impossible. The ischaemic lesions found are described in detail, as are the instances of localized subarachnoid collections of blood (subarachnoid haematomata) which may well be important in the production of ischaemic lesions and intracerebral haemorrhage. Mention is also made of granular cell degeneration of the cerebellum which is uncommon and probably a terminal event.

Method of Investigation

At necropsy the brain was examined externally only, and the bleeding aneurysm located where this was possible without disturbing the blood lying in the fissures, or between the lobes. After fixation for one to two months in formol-saline, the basal vessels were removed from the specimen. The brain was then cut into numerous slices and the distribution of blood in the sulci and fissures noted; material for histology was taken from many areas of the cerebral cortex, basal ganglia, brain-stem, and cerebellum. Particular attention was paid to any area suggestive of infarction and sufficient sections were taken to map out the area involved.

Haematoxylin and eosin staining was used routinely, but Nissl, Mallory's phosphotungstic-acid-haematoxylin, Holzer, myelin, neurofibrillary, and fat stains were used where needed.

Results

Multiple aneurysms were found in 14 cases; in one of these, many small aneurysms were present on an angiomatous malformation in the Sylvian fissure. In a further case, a single aneurysm on an anterior cerebral artery was associated with an angioma in the frontal lobe on the same side. Thus, in 15 out of 32 cases (over 46%), multiple possible sources of bleeding existed. This is a higher percentage of multiple aneurysms than usually reported. Robertson (1949) found 5%, Bigelow (1955) 10%, Wilson *et al.* (1954) 19%, and Richardson and Hyland (1941) 25%. The true figure may not be as high as in this short series, but it will be far higher than is usually reported. Small aneurysms arising on the superior aspect of the circle of Willis will only be detected after the vessels have been dissected off the brain.

The ruptured aneurysms were, in all but one instance, situated anteriorly on the circle of Willis or its branches. Commonly, two vessels were

involved by the aneurysm arising at a bifurcation, a fact of surgical importance and one which may influence the distribution of the ischaemic lesions. Subdural haematomas were seen twice from ruptured internal carotid aneurysms, but on neither occasion were they large enough to be of practical importance. Some surface subarachnoid haemorrhage was visible in all but one case, and, in this, separation of the frontal lobes revealed a massive haemorrhage. In 14 the surface bleeding was slight, but in 17 it was generalized, and at least 2–3 mm. thick over some portion of the brain. In only three of the 32 cases was massive generalized subarachnoid bleeding the only significant finding. The other 29 (90%) had additional lesions.

Localized Collections of Blood in Subarachnoid Space (Subarachnoid Haematomata). — The impression that, at most, a little blood clot is met with in the subarachnoid space (Falconer, 1954) is shown to be incorrect by the examination of brains which have been fixed without disturbance of the sulci or fissures. With or without generalized subarachnoid bleeding, sulci close to the aneurysm are often widened by blood. This may spread in the depth of the sulci

FIG. 1.—A massive collection of blood confined to the Sylvian fissure and depths of neighbouring sulci.

FIG. 2.—Blood in the Sylvian fissure with rupture down a sulcus on the insular cortex. Infarction surrounding distended neighbouring sulcus.

for considerable distances and be from 0.5 to 1.5 cm. thick and accompanied by infarction in the surrounding cortex. Subarachnoid haematomata are particularly liable to collect between the frontal lobes from ruptured aneurysms on the anterior communicating and anterior cerebral arteries, and in the Sylvian fissures from ruptured middle cerebral aneurysms. In these situations, the blood may be confined to the subarachnoid space, or may rupture into the cerebral tissue, whilst little escapes on to the brain surface. Large haematomata act as space-occupying masses, and are not infrequently associated with infarction.

In this series, large haematomata, totally or partially confined to the subarachnoid space, were present in nine (28%) brains.

Three very large collections were seen in the Sylvian fissure from ruptured middle cerebral aneurysms, one (Fig. 1) being entirely confined to the fissure and causing death by acting as a space-occupying lesion. The white matter of the temporal lobe was markedly oedematous and foci of acute neuronal degeneration were present in the insular cortex. In the second instance (Fig. 2) a massive clot was present in the Sylvian fissure

and had caused limited intracerebral bleeding by breaching the deepest limit of an insular sulcus. The blood also greatly widened a neighbouring sulcus. The insular and much of the temporal cortex was infarcted. Again, little surface bleeding was present. In the third case a mass of blood, 6 cm. long and 3 cm. wide, in the Sylvian fissure had ruptured into the postero-superior aspect of the insula, producing intracerebral and some intraventricular bleeding; pontine and peduncular haemorrhages, the result of the supratentorial mass, were present. The aneurysm was partly embedded in cerebral tissue but had not ruptured directly into the brain, the breach of the insular cortex being far posterior to the aneurysm. Small areas of acute infarction were present in the cortex of the middle and superior temporal gyri.

Six large subarachnoid haematomata were associated with ruptured aneurysms on the anterior cerebral or anterior communicating arteries. From these sites the blood may burrow into the sulci on the medial and inferior surfaces of the frontal lobes, or collect between the frontal lobes. If this occurs the thin rostrum of the corpus callosum may rupture with resulting intraventricular bleeding, as seen in Fig. 3. In this patient subarachnoid bleeding was present long before the intraventricular haemorrhage, since, three weeks before death, the patient had recurrent headaches with blood-stained cerebrospinal fluid, before becoming deeply unconscious a few days before death. Further, small areas of infarction of some standing were present in the frontal lobes, for new blood vessel formation and astrocytic proliferation had occurred and the peripheral parts of the blood clot itself were being organized.

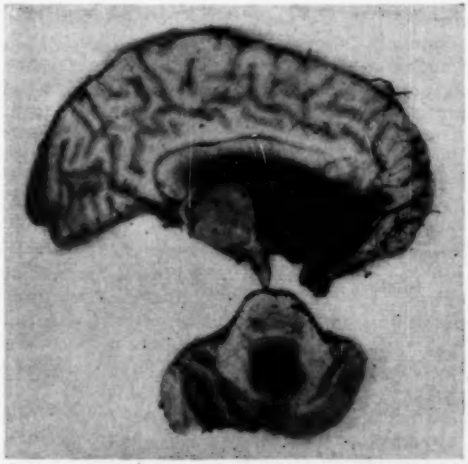

FIG. 3.—A massive collection of blood burrowing into sulci on the inferomedial surface of the frontal lobe and causing intraventricular haemorrhage by tearing the rostrum of the corpus callosum.

A second case presented almost identical pathological findings.

In the third case a blood clot, 3 cm. across, separated the frontal lobes, opening up the sulci on the medial surface on the left side, with numerous narrow tracks of blood into the hemisphere. This, like Robertson's (1949) similar case, was accompanied by infarction over the distribution of both anterior cerebral arteries. The fourth case presented a similar blood clot between the frontal lobes with necrosis limited to the frontal lobes in the immediate vicinity of the clot. In the fifth case much blood was present in the basal cisterns, but the Sylvian fissures and parieto-temporal sulci had been greatly widened, and blood had extended into the olfactory sulcus from below, producing what at first sight appeared to be an intracerebral haemorrhage (Fig. 4). The triangular-shaped blood clot was, however, almost completely surrounded by grey matter and was confined to the sulcus except at its posterior limit, where it had slightly extended into the brain. Infarction of the insular cortex and putamen was present. The sixth case showed an extension of blood over the superior surface of the infarcted and collapsed corpus callosum (Fig. 5), and there was massive bilateral infarction of anterior cerebral artery distribution. An attempt to clip the aneurysm, five days before death, was not responsible for the findings, for the histology was that of infarction of several weeks' standing and the diagnosis of frontal lobe infarction was suggested before operation. Similar cases have been seen by Robertson (1949) and Maloney (1958).

In two other instances the olfactory sulcus has been markedly distended by blood, and clot more than 1 cm. wide has been seen in the Sylvian fissure also twice. In a further case, from rupture of an internal carotid aneurysm, blood had tracked into, and greatly distended, many sulci on the inferomedial surface of the hemisphere on the side of the aneurysm, with little trace of bleeding over the gyri.

Intracerebral and Intraventricular Haemorrhage.—Bleeding into the brain occurred in 17 of the 32 cases (53%), and in 12 of these massive bleeding reached the ventricles. The route to the ventricles was through the rostrum of the corpus

FIG. 4.—A massive collection of blood in a greatly widened olfactory sulcus; much blood in Sylvian fissure and depths of neighbouring sulci. Infarction of putamen.

callosum in seven, the insular cortex in one, and the frontal lobe to the anterior horn in four. In three cases where the rostrum was torn, haematomata were present in the frontal lobe. Of the five instances of intracerebral haematomata without rupture into the ventricles, three were into the basal ganglia through the insular cortex, one into the temporal lobe, and one into the frontal lobe. An important point in relation to these 17 instances is that in only six was the aneurysm adherent to or embedded in cerebral tissue, and the bleeding into the brain was therefore not direct.

A little blood was present in the ventricles in a further six instances; in two of these it had reached the anterior horn of the lateral ventricle via narrow haemorrhagic tracks through the cerebral substance, but in four blood had apparently reached the ventricles through the foramina of the fourth ventricle.

The percentage of intracerebral haemorrhage in this series is somewhat lower than the 60–75 which is usually recorded, but it would have been considerably higher (68%) if subarachnoid haematomata had not been separated from intracerebral bleeding. One must also distinguish massive intracerebral bleeding from the linear haemorrhages which are common in the neighbourhood of the ruptured aneurysm. These latter, histologically, are perivascular and often run at right angles to the cerebral surface, and when numerous they are usually accompanied by parenchymatous degeneration. Their appearances strongly suggest that they result from blood tracking along the Virchow-Robin spaces into the cerebral tissue itself, forming linear intracerebral haemorrhages. It is clearly possible that coalescence of numerous haemorrhages produced in this fashion may result in a large haematoma.

Massive bleeding into the ventricles usually causes death in less than six hours (Bebin and Currier, 1957). Blood cannot escape rapidly from the ventricles (Symonds, 1924), and great dilatation commonly follows intraventricular bleeding. The fourth ventricle is often dilated to several times its normal size, with consequent distortion and pressure on pontine, medullary, and cerebellar structures. Haemorrhages, oedema, and occasionally actual infarction in the roof of the fourth ventricle result, and, less frequently, lesions in the medulla and pons. Subependymal haemorrhages also occur in the lateral ventricles, and less commonly in the peri-aqueductal tissues and anterior hypothalamus. Hypothalamic haemorrhages and necrosis are common with rupture of the rostrum, and in this series have been seen nine times; in eight the lesions were in the anterior hypothalamus. In six instances this hypothalamic damage was associated with massive intraventricular haemorrhage; in the other three instances the ruptured aneurysm was twice situated on the anterior cerebral and once on the internal carotid artery.

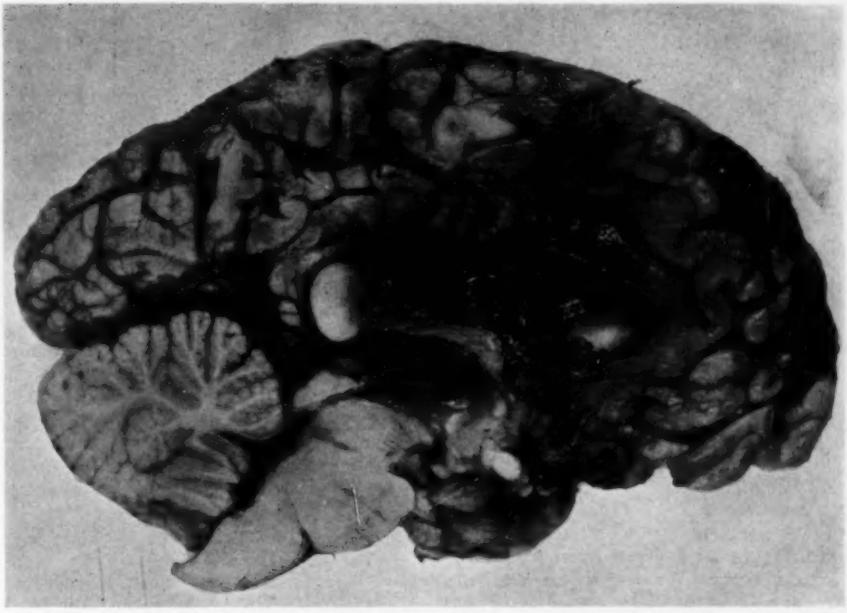

FIG. 5.—Blood clot lying over infarcted corpus callosum. Infarction of medial surface of anterior half of hemisphere.

Haemorrhages or degeneration, separately, or together, in the pons or cerebral peduncles, have been seen nine times, and in all but one instance, where infarction in the distribution of the posterior cerebral artery was present, they were considered to be due to increased supratentorial pressure. In four instances they accompanied massive intraventricular haemorrhage, in three subarachnoid bleeding without intracerebral bleeding, and in one massive infarction with great swelling of a hemisphere.

Cerebral Infarction. — Localized degeneration and gliosis up to 1 cm. in depth is almost invariable around aneurysms embedded in cerebral tissue. This lesion may be asymptomatic and found in people who die from a single rapidly fatal haemorrhage. In addition, ischaemic lesions, varying from microscopic foci to massive infarctions, are very common. In this series 25 of the 32 brains examined (78%) showed ischaemic lesions, and in 13 (40%) these were massive. The smaller lesions are often multiple, and cortical foci up to 1 cm. across may be separated by areas of normal brain, and in the fixed specimen appear as pale, irregularly round areas in the cortex, to which, if numerous, they impart a "moth-eaten" appearance. Similar, scattered foci are infrequently seen in the deep white matter, and occasionally, as reported by Falconer (1954), they occur in the depths of the sulci. If the foci are few, they are usually in the immediate neighbourhood of the ruptured aneurysm. They are particularly common on the medial surface of the hemisphere with ruptured aneurysms on or near the anterior communicating vessels. Four instances of such lesions have been seen in this series. In ruptured middle cerebral aneurysms similar foci occur on the insular cortex but also in the parietal or temporal lobe well away from the source of bleeding. With ruptured internal carotid aneurysms ischaemic foci may occur in the territory of both the middle and anterior cerebral arteries.

Of the 13 major infarctions, four involved the cortical territory of the anterior cerebral artery, on three occasions bilaterally. In two examples of bilateral infarction, the aneurysm was on the anterior communicating artery; in the third it was on the internal carotid, and patchy infarction of middle cerebral artery distribution was also present. In these three cases the cortex and underlying white matter was to a large extent destroyed in all sections from the medial surface of both hemispheres as far as the occipito-parietal

sulcus and of the corpus callosum. In the fourth case the aneurysm was on the anterior communicating artery and one frontal lobe showed massive infarction, the other only scattered ischaemic foci. In three of the above infarction was limited to the cortical supply of the artery, but in one areas of infarction in the head of the caudate nucleus and anterior limb of the internal capsule indicated involvement of central branches. In two of the above four instances the infarction was associated with subarachnoid haematomata, once between the anterior frontal lobes and once over the corpus callosum. In the other two, massive intraventricular haemorrhage had occurred through rupture of the rostrum, and in one of these a large haematoma filled the olfactory sulcus. Since massive intraventricular rupture is probably rapidly fatal, it is likely in these cases that considerable haemorrhage anterior to the rostrum was present for some time before its rupture.

In eight cases there was infarction of a large area of brain supplied by the middle cerebral artery. In three the aneurysm was on the middle cerebral artery on the same side as the infarction and in three it was on the internal carotid on that side. In two it was on the anterior communicating artery; in one of these a haematoma occupied the frontal lobe. In both cases the only infarction was in the territory of the middle cerebral artery. In three of the eight cases almost all the cortex supplied by the middle cerebral artery was infarcted, and in two of these the territory of the posterior cerebral artery was also involved (Fig. 6). In the other five cases a considerable area of cortex supplied by the middle cerebral artery was infarcted; in two the insular was most severely involved. A considerable depth of white matter was involved in these infarctions, usually reaching the lateral ventricle; in two instances foci of softening throughout the centrum semiovale were more obvious to the naked eye than cortical lesions, though the latter were extensive microscopically. Foci of infarction were found in the basal ganglia only once, indicating that the central branches of the middle cerebral artery usually escape involvement. In four of these eight instances a layer of blood more than 1 cm. thick was present in the Sylvian fissure. Two of the remaining four had an intracerebral haematoma, one in the frontal lobe, the other in the anterior temporal lobe.

One instance of infarction of the entire territory of the posterior cerebral artery alone was seen, this associated with a ruptured aneurysm involving the basilar and posterior cerebral arteries. Only moderate bleeding around the ruptured aneurysm was present. The two instances of posterior cerebral artery infarction associated with middle cerebral artery lesions were probably the result of supratentorial pressure from the bleeding and from swelling of the hemisphere from the middle cerebral lesion. Feigin (1955) reported two similar cases.

FIG. 6.—Infarction of middle and posterior cerebral artery territory from rupture of an aneurysm on the middle cerebral artery.

In most instances the age of the infarction, based on histological appearances, corresponded approximately to the time of a major clinical episode. Two patients dying less than 24 hours after a single haemorrhage showed early, massive infarction, with patchy coagulative necrosis, numerous haemorrhages and oedema, and shrinkage of neurones in the involved tissue. A subject dying after three days showed great oedema of both frontal lobes with disintegration of the majority of neurones, myelin sheaths, and oligodendroglia. One man living for seven days showed great swelling of both frontal lobes with much neuronal destruction, increase of cellularity, new blood vessel formation, and well-marked softening of the white matter. Patients surviving longer than this showed progressively more

advanced softening of the white matter with distinct astrocytic proliferation after three weeks.

Of the 13 instances of major infarction, eight had only one episode of bleeding, but in the other five the course suggested recurrent haemorrhages, and in two of these the histology and symptomatology suggested that the infarction occurred with the second and not the initial haemorrhage. In a further case there were infarctions of obviously different ages, one related to the original bleeding six weeks before death, and the other to the final bleeding a few hours before death.

There was no evidence, from the clinical course of the patients in this series, that investigation or operation contributed to the development of infarction. Apart from the two cases eliminated because of surgery, two others underwent major operative procedures. In one, no infarction was present; in the other, frontal lobe infarction was correctly diagnosed before the operation. Two cases had exploratory burr holes; one had an infarction, but the operation was performed immediately before death and could not have produced the ischaemic lesion.

Eighteen cases received no treatment or only antibiotics. In this group there were four major infarctions. In the treated group five showed clinical evidence of a cerebral lesion which proved to be an infarction, before treatment or investigation was begun. Therefore, in nine cases a major ischaemic lesion developed independently of treatment.

Ten of the treated cases were investigated by angiography without adverse effects. Four cases with major infarctions received, for the treatment of decerebrate rigidity or multiple convulsions, medication capable of producing hypotension, and in one of these a severe episode of hypotension was recorded. Possibly, therefore, the ischaemic lesion in these patients was influenced by medication. There is no way of determining this; all four were gravely ill when treatment was begun, and possibly already had ischaemic lesions. But, clearly, hypotension in a patient with cerebral ischaemia is likely to increase the severity of the lesion.

Degeneration of Granular Layer of Cerebellum. — Four instances of degeneration of the granular layer of the cerebellum were seen. The histological features in these cases were very similar to those described by Leigh and Meyer (1949) in association with visceral carcinomata and hypoglycaemic coma; they also mentioned its occurrence in a case of subarachnoid haemorrhage. The lesions varied from necrosis of all the nucleated elements in the granular layer to patchy severe destruction of the granular layer in which Golgi type 2 cells were preserved. In one case severe destruction of the granular layer was present in all sections; the other three showed patchy destruction, one in all areas examined, the other two mainly in the dorso-medial folia. The Bergmann glia appeared unaffected, and the Purkinje cells were not reduced in number though, in one case, some showed acute chromatolysis. There was no evidence of reaction to the necrotic foci, and degeneration of myelin and neurofibrils, glial proliferation, or excess of lipoids could not be demonstrated. No changes were found in the dentate nucleus or the inferior olives.

The ruptured aneurysm in three of these cases was on the internal carotid artery and in the fourth on the anterior communicating artery. The bleeding was massive in three and slight in the fourth.

The lack of reaction to the degeneration suggests it was a terminal event. All four patients had an episode of subarachnoid bleeding three to seven weeks before death, with partial or complete recovery. All four died suddenly from a further bleeding at the end of this period of convalescence. Three had been in hospital after the original bleeding, and no evidence of cerebellar abnormality was noted.

Discussion

A variety of pathological lesions follows rupture of an intracranial aneurysm. With massive bleeding, death may occur quickly, and little be found beyond blood in the subarachnoid space or brain. In other cases bleeding leads to the development of subarachnoid or intracerebral haematomata which, acting as space-occupying lesions, result in temporal herniation, lesions in the brain-stem, and occasionally ischaemia of the occipital and temporal lobes and posterior thalamus. All these lesions have been seen in this series, and massive infarction with brain swelling can produce them. Ruptured anterior cerebral, anterior communicating, and middle cerebral aneurysms are particularly likely to produce haematomata between the frontal lobes or in the Sylvian fissure. From internal carotid aneurysms, blood may track into the Sylvian fissures, and from any aneurysm groups of sulci may be

opened out by tracking blood. Blood may also spread down the Virchow-Robin spaces into the brain, producing perivascular haemorrhages which often extend into the white matter. When the brain is sufficiently stretched by a localized collection intracerebral bleeding occurs. This process is probably more common than the rupture directly into the cerebral tissue of an aneurysm which has become embedded in, or adherent to, the brain. Adhesions in the subarachnoid space, resulting from previous haemorrhage, would further hinder the escape of blood from sulci or fissures and increase the likelihood of intracranial rupture; the delicate rostrum of the corpus callosum is especially liable to be torn by a neighbouring haematoma.

With rupture of any aneurysm, ischaemic damage to some portion of the brain is highly likely and massive infarction is not uncommon.

In this series no instance of a thrombosed major artery has been seen. Embolism from the aneurysmal sac is an equally unlikely cause of the infarction, for many involve the entire territory of an artery and the clot would clearly have to lodge in the main stem of the artery where its detection would be easy. Small peripheral emboli could account for scattered infarctions, but in many hundreds of sections examined an occluded cortical artery has been seen only once.

Three other possibilities exist to account for ischaemic lesions. First, localized collections of blood could, by stretching or kinking, greatly narrow the vessels (Johnson, Potter, and Reid, 1958). Second, a large mass of blood stretching a major vessel may tear small branches passing from the parent vessel to the brain. This would deprive small areas of brain of their blood supply and provide further sources of bleeding into the subarachnoid space or brain. Either or both of these mechanisms might account for scattered as well as major infarctions, and could be responsible for ischaemic lesions even when no localized collections are found at necropsy; stretching of vessels by a haematoma may lead to irreversible ischaemic damage, but if the blood later escapes into the generalized subarachnoid space or ventricles, no local collection will be found at necropsy.

The third possibility is that spasm may cause the ischaemic lesions. The radiological evidence that spasm occurs after subarachnoid haemorrhage is strong (Ecker and Riemenschneider, 1951; Norlén and Olivecrona, 1953). In peripheral arteries, stretching is a powerful stimulus to spasm (Simmons, 1956), but the same cannot be said with certainty of the cerebral vessels. Localized blanching of the cerebral vessels may follow the production of brain swelling from intracerebral injection of saline (Wolff, 1936), and Byrom (1954) attributed focal spasm during experimental window insertion to stretching of the affected vessels. In the latter's experiments on rats local pressure on arteries did not result in arterial contraction, though rubbing vessels (Florey, 1925) produced spasm which varied with the animal used. The latter also observed spasm on either side of a point of rupture, though it was insufficient to stop bleeding entirely.

Therefore, three mechanisms exist which might lead to ischaemic lesions; these are stretching or kinking of vessels, tearing of small vessels, and spasm possibly induced by stretching. Sufficient evidence to incriminate one or more of these mechanisms is not at present available, but all of them might be produced by haematomata, and these are demonstrable in many instances.

When intraventricular bleeding occurs slowly the blood may, presumably, escape from the ventricles sufficiently quickly to prevent their gross distension. Massive bleeding with great dilatation of the ventricles is, however, common and clearly the rate of entry of blood into the ventricles is then greater than its speed of exit through the foramina. The original bleeding need not be rapid for this to occur. It is more likely that rapidly fatal intraventricular haemorrhage results from rupture of an intracerebral or subarachnoid haematoma into the ventricular system, the haematoma often having collected slowly. Obstruction to the escape of blood from the ventricles can occur from compression of the aqueduct after temporal herniation (Smyth and Henderson, 1938), but no instance of this has been noted in this series.

No explanation can be offered for the granule-cell degeneration of the cerebellum, and it would appear to be a terminal phenomenon in the cases reported here.

Patients who survive rupture of an intracranial aneurysm without sequelae presumably escape major infarction and large intracerebral or subarachnoid haematomas. When recovery is complete the bleeding will have been either small or from a site where blood could freely escape into the generalized subarachnoid space. But in Walton's series (1956), only about one third were so fortunate, and the remainder had residual abnormalities. The cerebral lesions in these cases

probably only differed in degree from those in the fatal cases. Intracerebral and subarachnoid haematomas will be largely absorbed with time, and ischaemia of varying degree probably accounts for most of the disabilities, though only a careful study of such cases would settle this point.

It may be inappropriate to discuss treatment on the basis of the findings in these fatal cases, but two points stand out. Draining the spinal subarachnoid space can only be of value when the blood has ready access from the bleeding site to the basal cisterns. When haematomata have formed, draining the spinal theca will be futile and dangerous. Secondly, some cases have subarachnoid or intracerebral collections which require immediate surgery. Attempts to deal with the aneurysm within a few hours of haemorrhage have frequently had dire results, but there is little to be lost in attempting simple drainage of entrapped collections through burr holes in patients who otherwise appear certain to die. The knowledge that these large collections are usually in or between the frontal lobes, or in the Sylvian fissure or temporal lobes, should make attempted evacuation a reasonable possibility in patients otherwise considered hopeless. The surgical attack on the actual ruptured vessel could be considered if improvement followed direct drainage of the blood.

Summary

The findings in the brain in 32 fatal cases of ruptured intracranial aneurysm are reported.

Blood tends to collect between the frontal lobes, in the Sylvian fissure, and in the depths of sulci, and in these positions may form large haematomata. These may cause death by acting as space-occupying lesions and may lead to intracerebral and intraventricular haemorrhage. They are probably of importance in the development of infarction.

Ischaemic cerebral lesions are very common and massive infarction was found in 40% in this series. Ischaemic lesions probably produce most of the residual disabilities in non-fatal subarachnoid haemorrhage. The possible mechanisms of production of ischaemic lesions are discussed.

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THE VALUE OF THE EXAMINATION OF THE CEREBROSPINAL FLUID IN THE DIAGNOSIS OF INTRACRANIAL TUMOURS

BY

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Examination of the lumbar cerebrospinal fluid in the diagnosis of cerebral tumour is less often resorted to than formerly, partly because in some instances there may be a risk of coning after lumbar puncture, but more especially because arteriography and ventriculography are now more frequently used for locating the tumour and examination of the ventricular fluid is often found to be more convenient. Nevertheless, if adequate precautions are taken, the examination of the lumbar cerebrospinal fluid is also not without its use in differential diagnosis.

In 1954 the authors read a paper at the 2nd International Congress of Clinical Pathology at Washington on some findings, including the presence of tumour cells, in the cerebrospinal fluid obtained from patients with cerebral tumours. Since then Locoge and Cumings (1958) have reviewed their results in over 12,000 fluids examined, and of these 835 were from patients with various types of intracranial tumour. The present paper records the findings in a total of 916 patients with histologically verified intracranial tumours of certain types seen at the National Hospital, Queen Square, between 1936 and 1957, and at Maida Vale between 1950 and 1957. Attention has been directed to the cell count and to the total protein content, with special reference to the finding of tumour cells and to the sugar content of the fluid.

Method and Material

Cerebrospinal fluid was obtained before operation and the cells counted in a Fuchs-Rosenthal chamber. The method of Mestrezat as given by Greenfield and Carmichael (1925) was used for the protein estimation. The sugar content was estimated by the method of Folin and Wu (Harrison, 1930).

During the past few years films have been prepared from a centrifuged deposit of cerebrospinal fluid in all likely cases of tumour, and these, after rapidly drying and fixing in methyl alcohol, have been stained by Leishman's method, which in our hands has yielded better results than Papanicolaou's method. The principal groups of tumours investigated are set out in Table I to some extent anatomically; thus the gliomata from the cerebral hemispheres come first, followed by the tumours of the cerebellum, acoustic neuromas, meningiomas, tumours of and around the pituitary, and finally metastatic carcinoma.

Results

Table I records the results of the cell count and the protein content in the fluids from patients with various tumours listed according to their site and nature.

It has been found in this series, as in that of Locoge and Cumings (1958), that pleocytosis is most commonly seen in gliomas.

On a percentage basis, however, there was little difference between the cell count in a malignant glioma (astrocytic glioma, Kernohan grading III and IV) and in metastatic carcinoma of the brain, for in both series the count was raised in 20% and exceeded 10 in just over 15% of cases. But the less rapidly growing astrocytic gliomas of the cerebrum (Kernohan grades I and II) showed appreciably lower figures, not substantially different from those found in a small series of cases of oligodendroglioma. Rapidly spreading tumours involving the corpus callosum or the optic chiasm were especially associated with high cell counts.

A raised count appears to be of serious import and suggests that the tumour is in contact with the ventricles; if, in addition, the fluid is tinged yellow, necrosis or haemorrhage should be suspected and this is usually indicative of malignancy.

As to the nature of the cells as seen in the counting chamber and in films stained by Leishman's method after fixation in methyl alcohol, they are usually neutrophils, lymphocytes, and histiocytes, including compound granular corpuscles. The presence of the latter two types

TABLE I
CELL COUNT AND PROTEIN CONTENT IN VARIOUS TUMOURS

Percentage of Cases with			Number of Cases	Variety of Tumour	Percentage of Cases with		
5 Cells or Over per c.mm.	10 Cells or Over per c.mm.	20 Cells or Over per c.mm.			50 mg. Protein/100 ml. or Over	100 mg. Protein/100 ml. or Over	200 mg. Protein/100 ml. or Over
				Tumours sited in cerebrum:			
6·7	6·7	6·7	15	Oligodendroglioma	40·0	26·7	Nil
8·9	5·1	3·8	79	Astrocytoma (Kernohan's grade I and II)	51·9	24·1	8·9
20·4	15·7	10·5	172	Glioblastoma (Kernohan's grade III and IV)	67·4	31·4	14·5
13·3	6·7	6·7	30	*Glioma pontis*	63·3	30·0	6·7
				Tumours sited in cerebellum:			
12·5	6·2	6·2	16	Astrocytoma	68·6	31·3	18·8
9·1	4·5	4·5	22	Haemangioblastoma	54·5	27·3	13·6
13·3	6·7	Nil	15	Medulloblastoma	46·6	6·7	6·7
4·0	Nil	,,	50	*Acoustic neuroma*	98·0	84·0	58·0
4·8	0·8	,,	126	*Meningioma*	73·8	33·3	7·9
				Tumours involving sella turcica:			
12·8	8·5	4·3	47	Chromophobe adenoma	74·5	38·3	6·4
Nil	Nil	Nil	10	Eosinophil adenoma	80·0	40·0	Nil
11·5	3·8	3·8	26	Craniopharyngioma	61·5	30·4	15·4
20·4	15·3	8·5	59	*Secondary carcinoma*	74·5	33·9	10·4

betokens probable necrosis in the tumour and the adjacent brain tissue, while a lymphocytic pleocytosis indicates a reaction on the part of the adjacent ependyma or pia arachnoid.

Tumour cells have been identified in 17 fluids, but only in the last six years or so has a serious search been made for such cells. An account of 13 of these cases will be given.

Astrocytes or glioblasts were only once identified in the spinal fluid and the tumour was subsequently proved to be an astrocytic glioma (Kernohan grade IV).

Case 1 (N.H. 62675).—A married woman of 40 years, under the care of Mr. Wylie McKissock, gave a two-month history of headache and vomiting associated with mental deterioration and followed by confusion and weakness of the legs for three weeks and one attack of unconsciousness 11 days before admission. On examination, long-standing papilloedema, but no other cranial nerve defects, was found. Some weakness and slight incoordination in the arm and face and a trace of aphasia were noted. Ventriculography showed a filling defect in the left anterior horn extending to an area of calcification in the left mid-frontal region. At operation a large astrocytoma grade IV (Professor W. Blackwood) was found and partly removed from the left frontal region. Necropsy confirmed the clinical findings. The left ventricular fluid contained 1,500 tumour cells per c.mm. (Fig. 1) and 240 leucocytes per c.mm. The protein was 170 mg. per 100 ml.

We have not identified tumour cells in the lumbar cerebrospinal fluid in cases of hemisphere ependymoma or oligodendroglioma, but in one instance of the latter where the tumour was located in the right frontal lobe, tumour cells resembling epithelial cells were found in the ventricular fluid.

Case 2 (M.V. 36241).—A woman aged 42 was admitted under the care of Dr. P. Sandifer with a history of lack of concentration, failing memory, confusion over a period of two months, and headache.

On admission she was found to be severely demented and resistive with a left hemiparesis maximum in the arm, early papilloedema, and probably left hemianopia. An accurate assessment of sensory changes was not possible. The E.E.G. indicated a deep-seated lesion in the right frontal lobe near the midline. The right ventricular fluid was blood-stained, with a faintly yellow supernatant fluid and 90 cells per c.mm., most of them being mononuclears with centrally placed nuclei and swollen cytoplasm. In the counting chamber both the nuclei and the cytoplasm of the cells were unusually refractile and prominent, and small clusters were occasionally seen. They gave the appearance of being epithelial cells. The erythrocyte count was 980 per c.mm. and the protein value 225 mg. per 100 ml. The left ventricular fluid showed cells of similar appearance which numbered 70 per c.mm. There were very few erythrocytes. The protein content was 70 mg. per 100 ml., but the supernatant fluid was faintly yellow.

At operation (Mr. Valentine Logue) an oligodendroglioma was found in the head of the right caudate nucleus extending out into the frontal lobe and also backwards.

There were no significant differences in the cell counts as between cerebellar astrocytomas, medulloblastomas, or glioma pontis, and in no instance of tumour in these situations were malignant cells identified in the fluid. The medulloblastoma, however, by reason of its situation and undifferentiated character could be

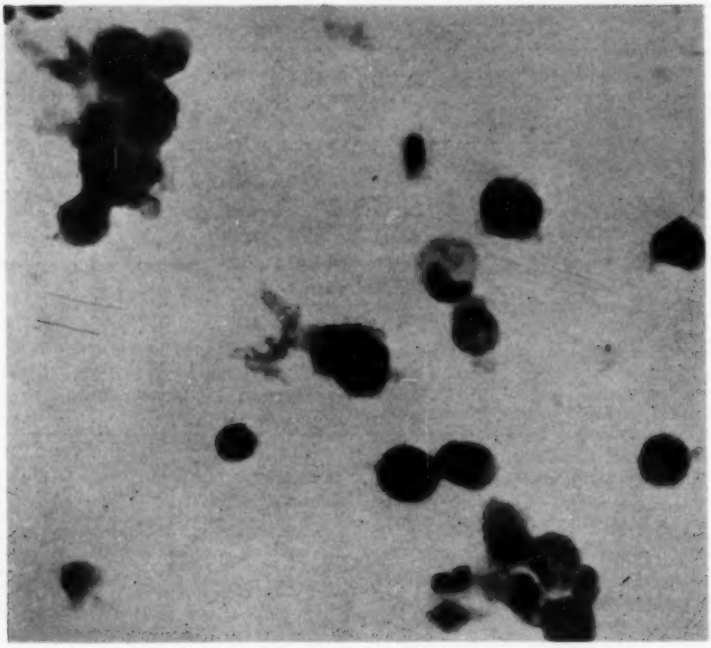

FIG. 1.—Case 1: Cells of astrocytic glioma (Kernohan grade IV), ×720.

expected frequently to shed tumour cells into the subarachnoid space, for in the absence of prophylactic radiation seeding frequently occurs along the cerebrospinal axis. The number of cases in the series, however, was but 15 and only in one did the count exceed 5 cells per c.mm. The cell count was raised in cerebellar haemangioblastoma less frequently than in astrocytoma or medulloblastoma.

In lumbar fluids from cases of chromophobe adenoma the cells have appeared to be mostly lymphocytes although some neutrophils have also been seen. In one instance, however, where the count was only 14 per c.mm., tumour cells were identified in the deposit and in their disposition were thought to bear some resemblance to the structure of a chromophobe adenoma.

Case 3 (M.V. 40801).—A man aged 27 was admitted under the care of Dr. S. Nevin with a two-year history of increasing irritability, loss of memory, obesity, and frontal headaches. More recent symptoms included drowsiness and deterioration in

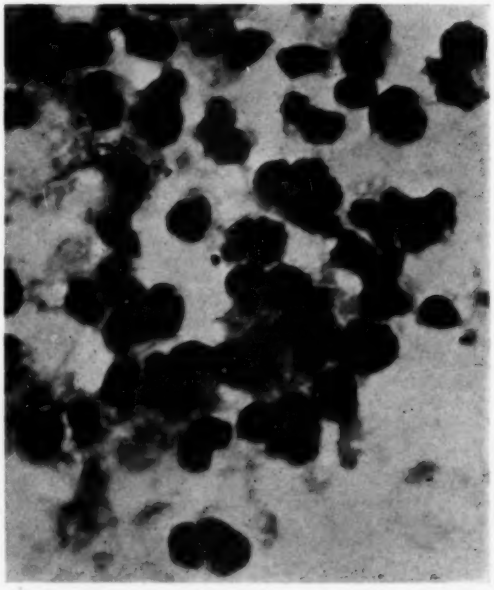

FIG. 2.—Case 3: Cells from chromophobe adenoma of the pituitary, ×850.

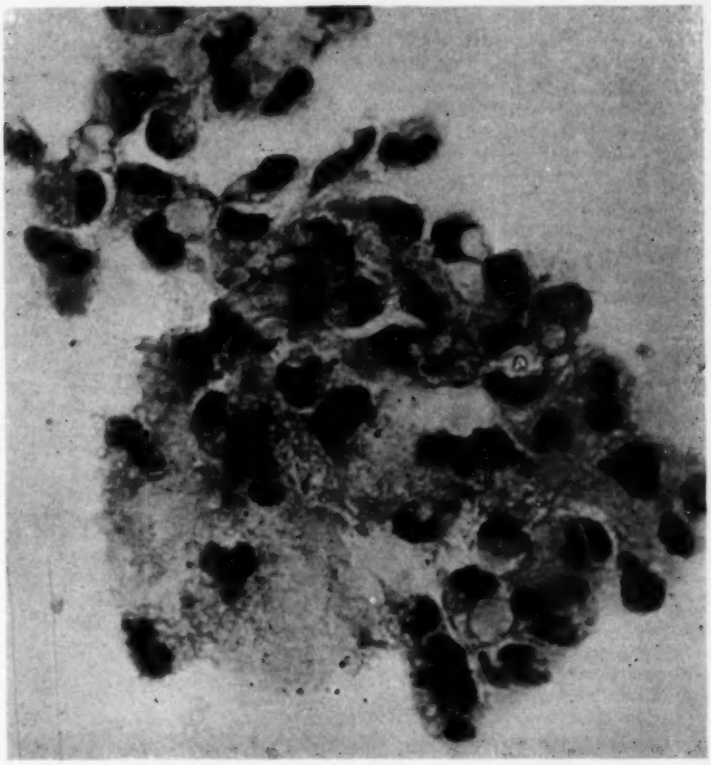

FIG. 3.—Case 4: A plaque of cells from a meningioma, ×750.

visual acuity. Positive signs consisted of a right lower temporal quadrantic defect, right hemiparesis, left-sided anosmia, and a right pupil which was larger than the left, with a poor convergence reflex.

Radiographs showed considerable enlargement of the pituitary fossa with undercutting of the anterior clinoids (Dr. D. Sutton). Lumbar puncture revealed a pale yellow fluid with 14 cells per c.mm., tumour cells being found in clusters (Fig. 2). The protein was 183 mg. per 100 ml. An air encephalogram confirmed the presence of a pituitary tumour. At operation Mr. V. Logue found a large purplish tumour lying mesially below the right optic nerve, which was running an almost vertical course, and also laterally. Histologically it was a chromophobe adenoma.

The cell count in suprasellar tumours was found to be similar to that of the chromophobe adenoma, being raised in 11.5% of instances of the former and in 12.8% of the latter. In a small series of 10 cases of eosinophilic adenoma the cell count was normal in all.

In acoustic neuromas and in meningiomas the cell count was rarely raised, but, in one instance of the latter, masses of cells were found and identified as tumour cells.

Case 4 (N.H. 2454).—A man of 54, under Sir Charles Symonds, had suffered for seven years from attacks of feeling as though he were losing consciousness associated with an inability to speak. When first examined in 1947 there was a slight facial weakness with increased arm jerks. Uncinate fits were considered a likely diagnosis. In 1954 he had intense pain on the left side of the head, tinnitus, dysphasia, dysgraphia, and some degree of impairment of comprehension. Motor power was good but tendon jerks were increased. There was slight papilloedema. The lumbar cerebrospinal fluid contained 1 cell per c.mm. and the protein was 140 mg. per 100 ml. (Fig. 3).

At operation (Mr. Wylie McKissock) a meningioma on the outer wing of the left sphenoid was found and removed. The patient remains well.

Carcinoma cells were probably present in most of the 20% of cases of secondary carcinoma of the brain in which the cell count was 5 per c.mm. or over, but only in a small proportion have we recognized them as such. Not all of the cells found in the cerebrospinal fluid in cases of carcinoma of the central nervous system were neoplastic; neither may the cell count be always raised. Secondary depositions of tumour around the subarachnoid space and ventricular space were particularly apt to cause a raised cell count and sometimes tumour cell desquamation. Tumour cells seem to be specially prolific when the cauda equina is secondarily involved by dissemination from higher up in the cerebrospinal axis.

The carcinoma cell is recognizable by reason of its bizarre character, its irregular and often massive nucleus, and its hyperchromatism. Cytoplasmic vacuolation is not infrequent, while mitosis is often observed. Vacuolation of the nucleus is less commonly found. Clusters of cells in which the irregularity in size of the individual cells can often be noted and multinucleate forms are common findings. A mucoid degeneration of the cytoplasm may occur with peripheral displacement of the nucleus, giving rise to "signet ring" forms. When such a change takes place in a multinucleated cell, the nuclei become scattered and displaced to the periphery in a mammillated fashion (Fig. 7).

In neurological hospitals a bronchial origin seems to be by far the most frequent cause of cerebral carcinomatosis.

It does not seem possible to identify the histological type of carcinoma from the appearance of the cells in the cerebrospinal fluid; nor is it always feasible to distinguish as between carcinoma and glioma cells.

The presence of mitosis must not in itself be accepted as evidence of malignancy, for just as endothelial cells may be encountered in pleural or peritoneal fluids in a stage of mitosis or in the form of a binucleated dividing cell so may they be found in cases of recovering meningitis. Carcinomatosis of the meninges bears a further resemblance to carcinomatosis of the pleural and peritoneal cavities because malignant cells, on account of their invasive propensity, may induce bleeding with subsequent discoloration of the fluid.

A few examples of the findings in cases of secondary carcinoma may be cited.

Case 5 (N.H. 64329).—A man of 44, under the care of Dr. M. Critchley, suffered from headache for eight weeks, vomiting for four weeks, and double vision for two weeks. He appeared to have lost weight. The pupils were unequal, the left being larger, and both reacted to light slowly. There was a left-sided ptosis and some loss of eye movements on that side. There was a slight loss of sensation on the right side of the face. No other physical signs were present apart from slight clubbing of the fingers and toes. Death occurred four weeks later without further signs or evidence of any primary tumour. No necropsy was permitted. The cerebrospinal fluid contained 790 cells per c.mm., of which 60% were carcinoma cells (Fig. 4); the protein was 900 mg. and the sugar 16 mg. per 100 ml.

Case 6 (M.V. 33241).—A housewife, aged 53, was admitted to hospital under the care of Dr. R. A. Henson with a six months' history of general irritability with buzzing noises in the ears and later giddiness. A month later she developed a frontal headache, a sense of pressure over the eyes, photophobia, and frequent vomiting. For the previous few weeks she had complained of increasing unsteadiness on her legs. There was no history of mental deterioration, but her speech was said to be slurred, especially at night. During the week before admission she had been noted to be disorientated and confused and had complained that "things did not make sense."

The physical signs indicated a midline posterior fossa tumour, but the history of dysphasia suggested the possibility of there being other tumours too, but no likely primary source for them could be found.

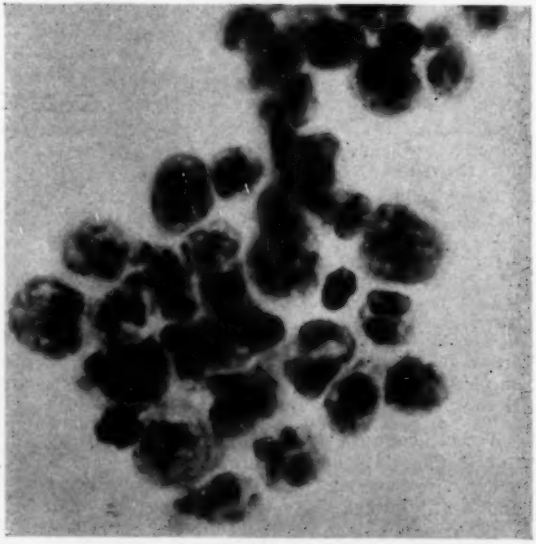

FIG. 4.—Case 5: A collection of carcinoma cells, ×950.

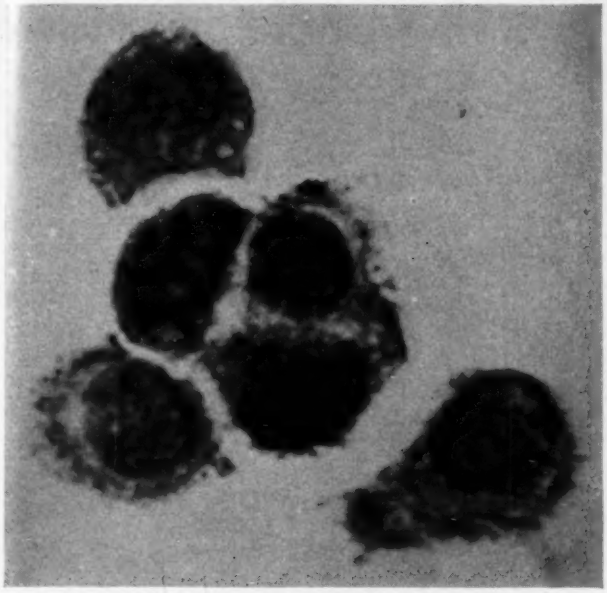

FIG. 5.—Case 6: Carcinoma cells (primary in lung), ×1,500.

Cerebrospinal fluid (2.5 ml.) removed by lumbar puncture was blood-stained, the supernatant layer after centrifugation being pale yellow. There were 21 cells (Fig. 5) per c.mm. (neutrophils 60%, lymphocytes 25%, and tumour cells 15%), chlorides 705 mg., and glucose 55 mg. per 100 ml. The Wassermann reaction was negative. The pressure was 210 mm. The fluid from the left ventricle was clear and colourless, but 45% of 22 cells were deemed to be neoplastic. The protein was 20 mg. per 100 ml. Ventriculography revealed no evidence of a space-occupying lesion.

In a subsequent lumbar fluid sample with a cell count of 93 per c.mm., of which one third were tumour cells, the glucose level was only 30 mg. per 100 ml. At no time was papilloedema observed and a repeat ventriculogram failed to show more than a slight degree of dilatation of the lateral ventricles. The haemoglobin, blood count, and E.S.R. were normal, but a search of a blood film revealed a promyelocyte and two erythroblasts. There was no radiological evidence of bony metastases and no primary tumour was located. Death occurred one month later.

At necropsy a small softened (squamoid) carcinoma was identified towards the periphery of the lower lobe of the right lung, with carcinomatosis of the leptomeninges. No other metastases were identified. Other pathological findings were healed rheumatic carditis, healed tuberculous adenitis of the mesentery, cholelithiasis, and early biliary cirrhosis.

Case 7 (M.V. 26360).—A cigarette smoker, aged 42, was admitted under the care of Dr. S. Nevin with a six weeks' history of a steadily progressing weakness of the right leg and tingling in the right calf. Within a week the symptoms had spread to the opposite side. Three weeks before admission he complained of aching in the back of the neck followed by pain behind the left eye and transient diplopia.

On examination, he was found to have signs consistent with a cauda equina lesion and, in addition, left-sided ptosis, diplopia on looking down, and left dysphasia. The blood count and the E.S.R. were both normal. Four cerebrospinal fluids were examined, with results seen in Table II.

Although this patient had a left hilar shadow at the time of the first lumbar puncture, tumour cells were not identified until the third fluid was examined.

Necropsy confirmed the presence of a bronchial oat-cell carcinoma in the left upper lobe with extensive deposits in the cauda equina which were thought to have spread from a metastasis involving the left caudate nucleus and choroid plexus. There were several other small metastases in the brain and also in the liver, thyroid, pancreas, kidneys, adrenals, left hilar, left subclavicular and bilateral cervical lymphatic nodes, and in the diaphragm.

TABLE II

RESULTS IN CASE 7

Date of specimen ..	6/2/52	21/2/52	24/3/52	17/4/52
Appearance ..	Colourless	Faintly yellow	Faintly yellow	Yellow
Cells per c.mm. ..	22	90	93	43
R.B.C.s per c.mm.	10	42	<10	2,794
Protein (mg. per 100 ml.)	200	360	200	360

Case 8 (M.V. 40245).—A man aged 34, with a variety of recent occupations, a liking for dog racing and an "intermittent" smoker, was admitted under the care of Dr. Helen Dimsdale with a six weeks' history of headaches, stiffness of the neck, and deterioration of vision. For the past two weeks he had had episodes of vomiting.

On examination, he was drowsy and confused, with a slurring dysarthria. There was gross papilloedema, nystagmus both to right and left, a complete left and partial right abducens paralysis, bilateral depression of the corneal reflex more on the left than on the right, left facial weakness, deafness on the left, and bilateral extensor responses but with limb hypotonia. There was bilateral ataxia.

In view of the possibility of an acoustic neuroma, he was transferred to the care of Mr. Valentine Logue. A right frontal burr hole yielded at a depth of 4 cm. clear and colourless fluid under a pressure of 100 mm. which revealed 7 cells and 393 erythrocytes per c.mm., a protein level of 18 mg. per 100 ml. and a glucose level of 70 mg. per 100 ml. On centrifuging, this slightly hazy fluid became clear and was colourless. The cells, apart from a very occasional neutrophil, included lymphocytes and what were thought at first to be arachnoidal cells. These cells had a clear cytoplasm and a rather pyknotic nucleus usually displaced to the periphery. The Lange and Wassermann reactions were negative. A ventriculogram suggested a communicating hydrocephalus, but no clear evidence was obtained of an expanding lesion.

The lumbar fluid three days after ventriculography contained 42 cells and 560 erythrocytes per c.mm., the supernatant layer being pale yellow. The cells consisted of 5% neutrophils, 35% lymphocytes, and 60% "arachnoidal" cells, some of which were actively dividing. The protein was 55 mg. and the glucose 55 mg. per 100 ml. Three days later the cell count was 48 per c.mm. and the cytology substantially the same, but the protein was now 310 mg. per 100 ml. and the glucose only 21 mg. per 100 ml. Several of

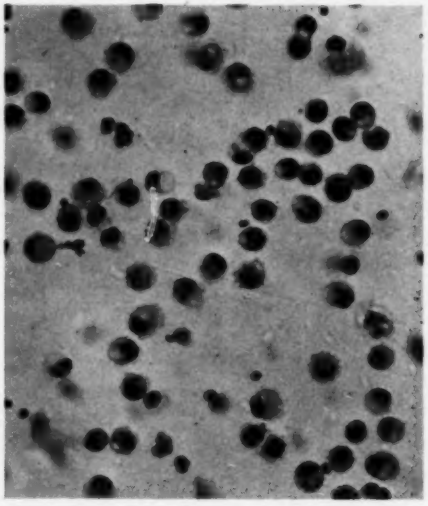

FIG. 6.—Case 8: Large numbers of carcinoma cells with signet ring forms, ×550.

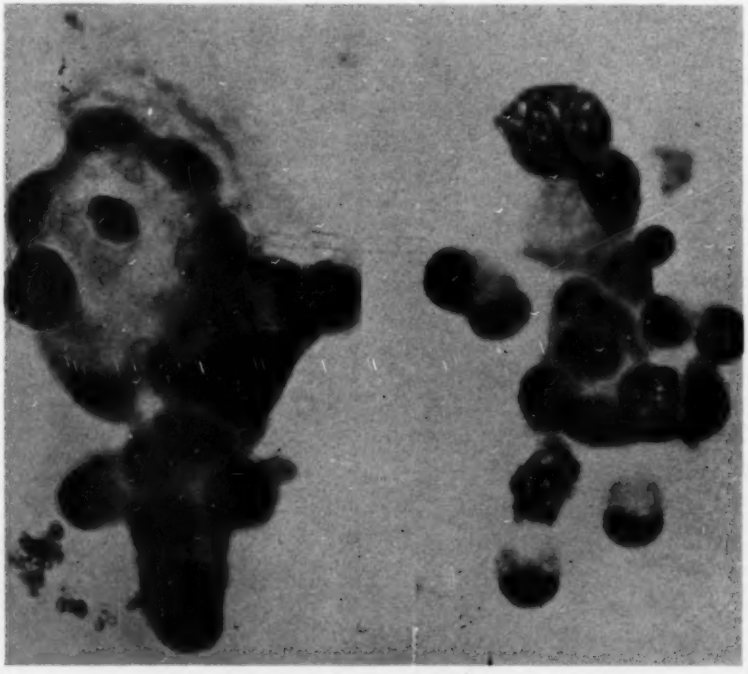

FIG. 7.—Case 8: A small clump of cells from the same case as Fig. 6, ×750.

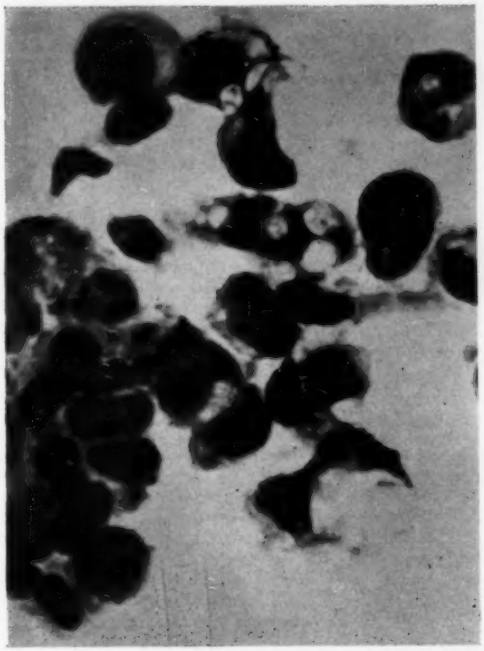

FIG. 8.—Case 9: A group of carcinoma cells, × 1,150 (primary in bronchus).

the cells were showing cytoplasmic vacuolation and some of the nuclei were larger than others and pyknotic. Carcinomatosis was by now regarded as the only probable diagnosis. As subsequent specimens were examined the cells become more certainly malignant and true signet ring forms were common (Figs. 6 and 7). Cells also appeared in clusters and there were many dividing forms. Two weeks after admission the cell count was 35 per c.mm. and the protein level 125 mg. per 100 ml.

Fluid in the right ventricle was by now faintly smoky with 23 cells per c.mm., mostly neoplastic, and 650 erythrocytes. The glucose level was 70 mg. per 100 ml., but varied from time to time, falling to 17 mg., and bore little reference to the tumour cell count. Death occurred one week later. At necropsy the brain showed an extensive meningeal carcinomatosis, but the primary source of the tumour was not identified. The carcinoma cells were at times arranged in imperfect alveoli and the cytoplasm was both plentiful and clear. At other times the cells resembled signet rings or they sometimes conformed to tall columnar epithelium.

Case 9 (M.V. 40915).—A widow, aged 58, was admitted under the care of Mr. Valentine Logue with a history of cough, pain, and dyspnoea extending over a period of 17 months. In another hospital bronchoscopy had revealed a probable carcinoma of the left lower lobe bronchus, but the histological findings were inconclusive. There was an associated basal pleural effusion. Further biopsies were again inconclusive, but pleural aspiration revealed cells suggestive of neoplasia.

Progressive drowsiness and loss of weight had developed during the two months before the present admission and a previous lumbar puncture had yielded a fluid with 60 cells ("lymphocytes") per c.mm. and 200 mg. protein per 100 ml. She was found to have, in addition to the marked drowsiness, left hemianopia, neck stiffness, increased left upper limb reflexes, and a right-sided grasp reflex.

Lumbar puncture revealed a slightly yellow fluid with 43 cells and 56 erythrocytes. The cells consisted of lymphocytes and tumour cells in about equal numbers (Fig. 8). The protein was 170 mg. and the glucose level 45 mg. per 100 ml. Two days later the cells were 55 per c.mm. and the glucose level 23 mg. per 100 ml. Equivocal pathology, similar to that found in the thoracic biopsies, was revealed in the piece of brain obtained from the ventricular needle.

At necropsy a massive sclerosing carcinoma was found in the mediastinum encircling the left lower bronchus. The brain showed a generalized opacity of the meninges due to the presence of atypical carcinoma cells, together with a metastasis in the right fornix invading the corpus callosum and involving the choroid plexus. There was, in addition, a widespread infiltration of the brain-stem by malignant cells. This case will be reported in greater detail.

Case 10 (M.V. 29621).—A machine operator, aged 44, was admitted under the care of Dr. P. Sandifer, with a six weeks' history of occipital pain associated with some stiffness of the neck. In the course of a few days the pain had spread over the left shoulder and down the arm and into the palm and all the fingers. Later, there had been a progressive weakness of the legs and a heavy numbness "like lead" associated with dysuria. Eventually a weakness of the right arm had developed.

On examination, he was noted to be a wasted man with early clubbing of the fingers. There was evidence of involvement of the roots of C5, C6, and C7 on both sides and of the cauda equina.

Cisternal puncture found a fluid faintly smoky and yellow. There were 31 cells per c.mm., 10% of which were malignant, and 853 erythrocytes. The protein was 130 mg. per 100 ml. The lumbar fluid was heavily blood-stained, the supernatant fluid being faintly yellow. There were only 48 cells counted against 10,240 erythrocytes, but they included a large proportion of malignant cells. The protein was 480 mg. per 100 ml. Radiographs revealed a rounded opacity at the left hilum suggestive of a carcinoma, while a myelogram showed multiple lateral indentations and filling defects in the region of C6–C7 on the right and C4–C5 on the left. He was referred for deep *x*-ray treatment and died in another hospital, no necropsy being sanctioned.

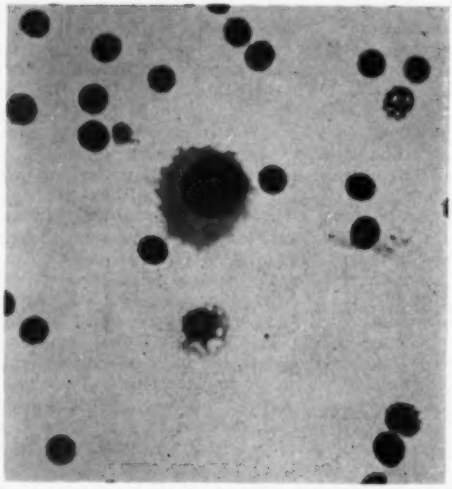

FIG. 9.—Case 11: A single melanoma cell (secondary melanotic metastasis), ×750.

Case 11 (N.H. 53106).—A married woman of 28, under the care of Mr. Wylie McKissock, had had a malignant melanoma removed from the left calf nine months earlier. A block dissection of glands had also been carried out. Five months before admission she had a normal child, but during the past four weeks had had lassitude and headache for five days. Later, vomiting and meningismus were prominent features. On examination, she was drowsy with neck stiffness, bilateral ptosis, and slight weakness in the arms and legs. Plantar responses were extensor and some hyperalgesia and hyperaesthesia of the legs were found. Ventriculography suggested a space-occupying lesion in the left frontal region. The right ventricular fluid showed 320 cells per c.mm., mostly tumour cells, suggestive of an origin from a melanoma and with a protein of 65 mg. per 100 ml. (Fig. 9).

The findings in two other patients will be mentioned, for tumour cells were found in the cerebrospinal fluid of both, although neither had a brain tumour.

Case 12 (M.V. 36211).—A warehouse worker, aged 64, under the care of Sir Russell Brain, had had several previous admissions to hospital on account of right temporal headache and symptoms and signs over a period of about four years, pointing to an expanding lesion compressing the anterior surface of the medulla and pons. The cerebrospinal fluid initially was under normal pressure with 4 cells per c.mm. and a protein content of 68 mg. per 100 ml. At the penultimate admission, however, the lumbar fluid had shown 11 cells ("small lymphocytes with occasional large mononuclears") and a protein content of 92 mg. per 100 ml. During his last admission the burr holes already present were re-entered and the fluid on both sides was found to be under considerable tension. That on the right was examined; the cells numbered fewer than 1 per c.mm. and the protein was 10 mg. per 100 ml. A catheter was inserted on this side. Before operation Mr. V. Logue tapped the left ventricle and the fluid was found to be faintly yellow with 22 cells per c.mm., a glucose level of 90 mg. per 100 ml., and a protein of 36 mg. per 100 ml. The cells were found to be neoplastic (Fig. 10), the most striking feature being a remarkable degree of cytoplasmic vacuolation in some of them. In addition there were occasional but noteworthy instances of intranucleal vacuolation (Dr. Helen C. Grant). Exploration revealed a large, whitish mucoid tumour seemingly arising from the basi-sphenoid and displacing the pons, medulla, and cord upwards and backwards. Histologically, it was a typical chordoma exhibiting the characteristic vacuolation both of the cytoplasm and of the nuclei.

The retrograde spread of the tumour cells had presumably been rendered possible on account of the high infratentorial pressure and the supratentorial drainage.

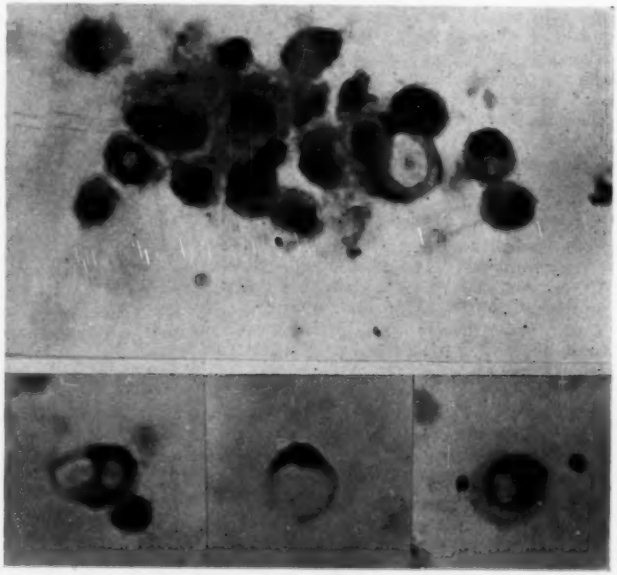

FIG. 10.—Case 12: Tumour cells from an intracranial chordoma, ×700.

FIG. 11.—Case 13: Typical plasma cells (spinal myeloma), ×750.

Case 13 (N.H. 53471).—A man of 43, under Mr. Harvey Jackson, had suffered from paraplegia for three months. He had signs suggesting a lesion in the region of the fourth thoracic vertebra with pressure on the spinal cord. The report of the radiograph (Dr. J. W. D. Bull) showed partial destruction of the left pedicle of D5. At operation portions of an extradural tumour and of the surrounding muscle were removed and shown to be infiltrated by a plasma cell myeloma (Professor W. Blackwood). A very small amount of cerebrospinal fluid was obtained and contained 16 cells per c.mm. including numerous plasma cells (Fig. 11).

So far as the protein values are concerned, the most striking feature is the constancy with which a raised value is obtained in cases of acoustic neuroma. In only one out of the 50 cases was the protein level below 50 mg. per 100 ml., while a figure of 200 mg. per 100 ml. was exceeded in 58%. The tumour which next most frequently yielded a fluid with a protein level in excess of 200 mg. per 100 ml. was a cerebellar astrocytoma, this value being exceeded in 18.8% of cases. However, the tumour which in its ability to give rise to a protein content of over 50 mg. per 100 ml. came next to the acoustic neuroma was the eosinophil adenoma of the pituitary at 80%, followed by the chromophobe adenoma and metastatic carcinoma both at 74.5%.

Discussion

In so far as the alterations in cell count and protein content in the cerebrospinal fluid are concerned, the findings in this group correspond closely with the results found by Locoge and Cumings.

Most interest, however, centres around the finding of neoplastic cells in the cerebrospinal fluid. Spriggs (1954) found malignant cells in the fluids of seven patients, and he collected from the literature 66 instances of malignant cells being recorded as present in the C.S.F., and, of these, 47 were in patients who were subsequently found to have secondary carcinomatosis.

The rarity with which tumour cells have been identified in the cerebrospinal fluid in cases of primary intracranial tumours is in contrast to the increasing frequency with which they have been recognized in meningeal carcinomatosis. We propose, therefore, to consider these groups separately.

Primary Intracranial Tumours. — The 21 reported instances in which malignant cells have been identified in the cerebrospinal fluid are recorded in Table III, and except where stated the fluids were obtained by lumbar puncture.

The cytological diagnosis of intracranial tumours other than carcinoma is a matter of some difficulty, because exfoliation seems to be much less frequent in the primary tumours, in spite of the fact that the cell count may often be raised, especially in malignant gliomas. The most important source of confusion is the macrophage, for abnormal degrees of swelling may be seen in this cell, especially in fluids which have been standing for any length of time. The advice of Cairns and Russell (1931) in this connexion should be heeded. The presence of mononuclear cells, they say, other than lymphocytes, should always arouse suspicion. But as with the carcinoma cell the criteria of recognition are the varying sizes of the cell, and in particular its nucleus, the presence of cytoplasmic vacuoles, and the degree of hyperchromatism. The presence of clumps of such cells is even more in favour of malignancy. A helpful point at times is an increased refractility. This was noted by Walt (1939) in his case of medulloblastoma and was useful in distinguishing these cells from lymphocytes. It was a feature noted in our case

TABLE III
REPORTED CASES OF MALIGNANT CELLS IDENTIFIED IN C.S.F.

Author	Date	Site and Nature of Tumour
Rindfleisch	1904	Tumour of third ventricle
Loeper and Crouzon	1906	"Sarcoma of occipital lobe"
Sicard and Gy ..	1908	"Sarcoma of dura"
Guillain and Verdun	1911	Juxtaventricular glioma
Fischer	1922	Part 2, case 1, multiple tumour Case 3, glioma
Cairns and Russell ..	1931	Pituitary adenoma
Ostertag	1932 1933	Spongioblastoma of cerebellum
Ford	1937	Medulloblastoma
Walt	1939	"
Schroeder *et al.* ..	1945	Glioma of cerebellum (ventricular fluid)
Platt	1951	Case 5, glioblastoma multiforme (ventricular fluid) Case 6, perithelial sarcoma of cerebellum (cisternal fluid) or medulloblastoma
Larson *et al.* ..	1953	Case 4, astroblastoma III at R. hemisphere Case 5, astrocytoma II at posterior parietal
Spriggs	1954	Case 5, medulloblastoma Case 6, glioblastoma multiforme involving pineal region and thalamus
McCormack *et al.* ..	1957	Case 20, medulloblastoma Case 21, pinealoma Case 22, glioblastoma multiforme
Adams	1958	Case 2, glioblastoma multiforme

of an oligodendroglioma (Case 2). In general, it is safer to admit that glioma cells cannot with confidence be distinguished from carcinoma cells: the only definite exceptions are the myeloma cell and the signet ring cell, which latter suggests a carcinoma with a primary site in the bronchus or the gastro-intestinal tract.

In some instances, in a hemisphere ependymoma, for instance, tumour cells could be expected with greater frequency in the ventricular than in the lumbar fluid, but the reverse would normally obtain in an ependymoma in the fourth ventricle or in a medulloblastoma. Astrocytic glioma as well as other forms of cerebral tumour not infrequently involve the ependymal walls of the lateral ventricles so that cytological examination of the ventricular fluid may often prove to be more rewarding than the lumbar fluid.

On two occasions cells have been found in the cerebrospinal fluid which appeared to resemble astrocytes, and in one instance in which the cell count was raised a diagnosis of astrocytoma was suggested, but at necropsy the disease proved to be multiple metastatic carcinoma.

Secondary Intracranial Tumours.—Carcinoma cells were first identified in the cerebrospinal fluid by Dufour (1904). Since the review of the literature by Spriggs (1954) a few other workers have recorded finding these cells. McCormack, Hazard, Belovich, and Gardner in 1957 found tumour cells in 17 cases, while Ravina, Pestel, and Thielen (1955) reported one case with tumour cells present but with no increase in total cell count. In the four cases described by Dodge, Sayre, and Svien (1952) the "large mononuclear cells" were not stated to be carcinoma cells as such, but in each instance the glucose content of the fluid was low. Others who have found carcinoma cells in the cerebrospinal fluid include Dattner (1952), Fischer-Williams, Bosanquet, and Daniel (1955), Reiseger (1956), and Heathfield and Williams (1956). As carcinoma cells have been found in fluids with no significant pleocytosis, a careful search of the centrifuged deposit will in such cases sometimes aid diagnosis. On the other hand, a carcinomatous involvement of the leptomeninges may excite a lymphocytic pleocytosis without any carcinoma cells appearing in the fluid. Strange (1952) found the cell count to be raised above 7 per c.mm. in 21 out of 30 fluids from 14 cases of meningeal carcinomatosis, but she makes no reference to the presence of malignant cells.

A low sugar content of the fluid was found by us on a number of occasions and this has also been observed in fluids when tumour cells had not been found during life, although necropsy later showed a carcinomatous meningitis. Other observers have commented on the low glucose content in cases of neoplastic disease of the meninges ever since Guillain and Verdun (1911) found sugar not detectable in the cerebrospinal fluid in one such case. This subject was discussed by Dodge *et al.* (1952), and they concluded that the fall was due in part to an acceleration of the metabolism of the tumour cells, and to a mechanical blocking of the blood–brain barrier so that crystalloids were not able to pass. Strange (1952) believed that carcinoma cells utilized glucose, but that stasis was also present. It seems to be generally agreed that when the glucose content is low the meningeal involvement is extensive. There is, however, no particular correlation between the number of tumour cells and the level of the glucose content in the fluid. Nevertheless, McCormack *et al.* (1957) advised an intensification of the search for malignant cells if the glucose content is found to be low, and our findings support this opinion. McCormack, Hazard, Gardner, and Klotz (1953) have reported figures as low as 11 and 13 mg. per 100 ml., McElligott and Smith (1958) record a value of 11 mg. per 100 ml., while Dodge *et al.* (1952) have noted a figure as low as 2.5 mg. per 100 ml., and both these last authors and Eaton (1952) report

cases in which no sugar was detectable. In 17 out of 18 cases of meningeal carcinomatosis studied by Berg (1953) the glucose content was below 50, while Strange in 18 recordings from seven cases found no value over 47 and one was as low as 9 mg. per 100 ml.

Other workers who have obtained figures for cerebrospinal fluid sugar of below 40 mg. per 100 ml. include Meissner (1953) in two cases, Murphy (1955) in one case, Jacobs and Richland (1951) in two cases, while Fischer-Williams *et al.* (1955) record a level of 50 mg. per 100 ml. and Reiseger (1956) a case with a level of 44 mg. per 100 ml.

Summary

The cerebrospinal fluid findings are reviewed in 916 patients with histologically verified intracranial tumours, the cell count and protein values being analysed according to the different categories of tumour, and their significance discussed.

Pleocytosis is most commonly found in glioma, but on a percentage basis it is as common in carcinomatosis of the brain. Tumour cells, however, are rarely found in gliomas but frequently in carcinoma.

Thirteen cases are reported in which tumour cells have been identified in the cerebrospinal fluid. They include instances of pituitary adenoma (only once previously described) and, for the first time, of oligodendroglioma, chordoma, and meningioma.

A falling glucose level is highly suggestive of carcinomatous meningitis and the search for tumour cells should be intensified. A cell count within the limits of normal does not exclude this diagnosis.

We wish to thank our colleagues mentioned in the text who have allowed us to use the records, and also the following for their technical assistance: Messrs. W. Shedden, B. Kaploon, J. A. Mills, and G. Cox.

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THREE CASES OF INTRACRANIAL VASCULAR MALFORMATIONS IN INFANTS

BY

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This paper is concerned with intracranial vascular malformations of hamartomatous origin and not with the relatively common aneurysmal, focal, intramural defects of the vessels of the brain. Such hamartomatous lesions have been well described (Cushing and Bailey, 1928 ; Lindau, 1931 ; Russell, 1931). They can be classified into three main types, namely (*a*) haemangioblastomas, (*b*) cavernous angiomas, and (*c*) racemose angiomas. Haemangioblastomas usually occur within the cerebellum and often form a cyst with only a small nodule of tumour in its wall. They present as cerebellar tumours and may be associated with similar lesions in the retina and with cysts of the pancreas, kidney, or liver (Lindau's disease). Cavernous angiomas are usually situated within the substance of the brain and are similar in structure to the common cutaneous haemangiomas ; occasionally they are associated with angiomatous lesions of the skin and other organs. The third variety is characterized by a mass of recognizable blood vessels in the pia arachnoid. These are variously named racemose angioma, cirsoid aneurysm, angioma venosum, angioma arteriale, or telangiectasis, depending largely upon the appearances at operation or necropsy. The three cases described in this communication had lesions of this third variety.

Case Reports

Case 1.—A baby girl was born at term and thrived for 42 hours. She then fed poorly and became restless with grunting respirations and rib recession. On the third day she developed cardiac failure. She was given digitalis but died when only 4 days old.

Necropsy Findings.—The body was that of a female baby with extremely livid skin and intense cyanosis of the lips and nail beds. The heart (41 g.) was almost twice the normal weight and both ventricles were dilated and hypertrophied. The common and internal carotid arteries were widened and thick walled. The caval veins and their tributaries were engorged and the lungs, liver, and kidneys were congested. The brain showed a dense mesh of small convoluted blood vessels in the pia arachnoid of the occipito-parietal regions, more marked on the left. The vein of Galen was greatly widened, measuring 1 cm. in diameter, and drained into a large venous sinus occupying much of the posterior part of the posterior fossa. The sinus filled the angle between the tentorium cerebelli and the squamous part of the occipital bone ; it received also the normal superior sagittal sinus, two large occipital sinuses, and hypoplastic straight and right transverse sinuses. The left transverse sinus was absent. Anteriorly the venous pool communicated with the sigmoid sinuses. On transecting the brain gross atrophy of the left occipital and parietal lobes, with thinning of the convolutions, was demonstrated (Fig. 1). These convolutions were of an opaque, rusty yellow colour and showed fine cystic degeneration, while the central white matter was soft and reddened and contained flecks of calcification. The left frontal and right occipital lobes were softened.

Histology.—All the viscera are intensely congested. The lungs show collapse, oedema, and occasional foci of inhalation pneumonia. There is fatty change in the periphery of the liver lobules, and the kidneys show fatty change in the convoluted tubules, with hyaline casts in the collecting tubules. The brain shows abundant, engorged blood vessels in the pia arachnoid of the left occipital and parietal lobes (Fig. 2) and, to a much lesser extent, of the other lobes. These blood vessels measure almost 1 mm. in diameter and often present a bizarre configuration in transverse section. Some are thin-walled veins and others are arteries with a muscular media and an internal elastic lamina. The elastic lamina is frequently duplicated and irregular and the intima often shows focal collagenous proliferation (Figs. 3 and 4). These vessels do not penetrate the brain substance but give rise to a plexus of dilated capillaries in the pia mater (Fig. 5). The brain substance contains many engorged capillaries especially around the many areas of cystic change. The brain tissue of the left frontal and right occipital lobes shows early infarction, much oedema, vascular engorgement, and some perivascular haemorrhage. Swelling and disintegration of nerve cells are seen, with proliferation of foamy microglia. In the left parietal and occipital lobes it is barely possible to distinguish any resemblances to normal architecture. The cortical

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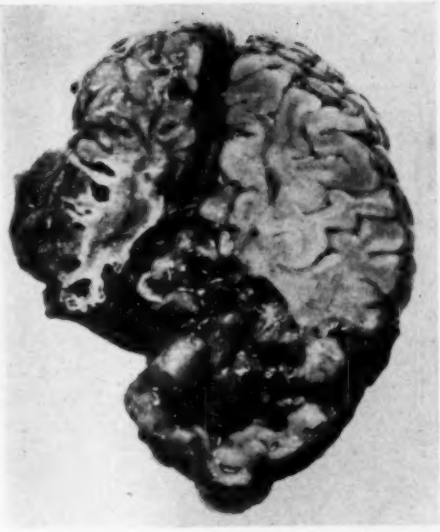

FIG. 1.—Case 1. Horizontal section of the brain showing atrophy of the left occipital and parietal lobes and thinning of the convolutions.

FIG. 2.—Case 1. Left occipital lobe showing many large engorged blood vessels in the pia arachnoid. There is thinning of the gyri and some focal calcification of the white matter. Haematoxylin and eosin, × 5.5.

FIG. 3.—Case 1. Left occipital lobe. The arteries have a bizarre configuration on section and show focal intimal thickening. The pia mater is thickened and contains abundant engorged capillaries. Elastica van Gieson, × 11.

FIG. 4.—Case 1. Left occipital lobe. Reduplication of the elastic lamina and focal intimal thickening in one of the arteries. Elastica van Gieson, × 100.

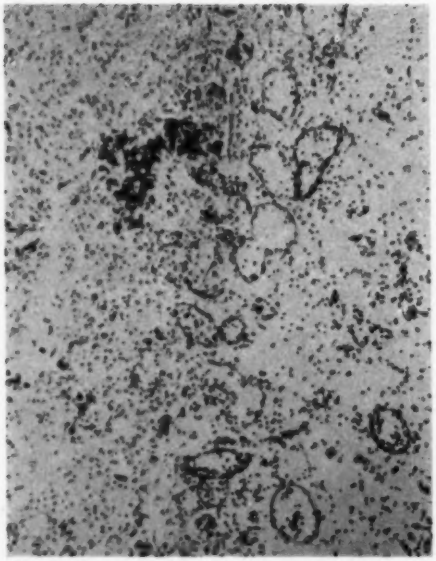

FIG. 5.—Case 1. Left occipital lobe. A capillary plexus in the pia mater. The underlying cortex contains foam cells and shows calcification. Haematoxylin and eosin, × 100.

region shows extensive microcystic change with surrounding cellular astrocytic gliosis. The white matter shows dense astrocytic gliosis containing many foci of calcification; in a few places capillary walls are calcified (Fig. 6). Both cortical and medullary zones show occasional foci of haemosiderin and contain abundant fat-filled microglia (Fig. 7). There is no thrombosis in any of the sections examined. Apart from hypertrophy, no other significant change is seen in the walls of the internal carotid arteries or internal jugular veins.

Case 2.—The patient was a full-term baby boy who was regarded as normal until 2½ weeks of age, when at a welfare clinic a cardiac murmur was heard. At 3 weeks of age he was breathless and cyanosed, the heart and liver were enlarged, and there was a loud apical systolic murmur. Pulsation was noticed in the neck. In spite of digitalis therapy, he died in severe congestive cardiac failure when 7 weeks old. On the day of death the pulse was collapsing and there was marked pulsation on both sides of the neck; the systolic blood pressure was 130 mm. Hg and the diastolic pressure could not be determined.

Necropsy Findings.—The body was that of a male infant of normal size for his age, whose lips and nail beds were cyanosed.

The heart (65 g.) was three times the normal weight, due to hypertrophy of both ventricles, which were dilated. Both common carotid arteries were wide and thick-walled. The jugular veins were engorged, the lungs oedematous and congested, and the liver and spleen congested.

FIG. 6.—Case 1. Left occipital lobe. Astrocytic gliosis of the white matter with focal calcification. Many vessel walls are calcified. Haematoxylin and eosin, × 70.

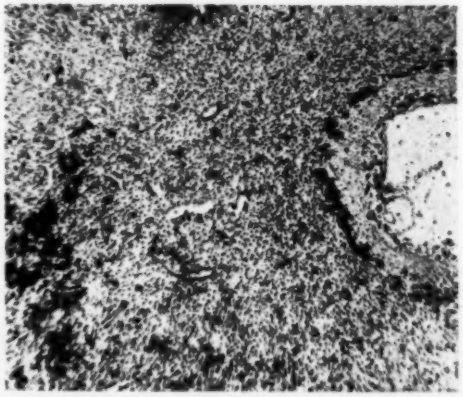

FIG. 7.—Case 1. Left occipital lobe. The base of a sulcus showing intracellular fat especially in the superficial cortex and subcortical white matter. Frozen section. Oil red O, × 18.

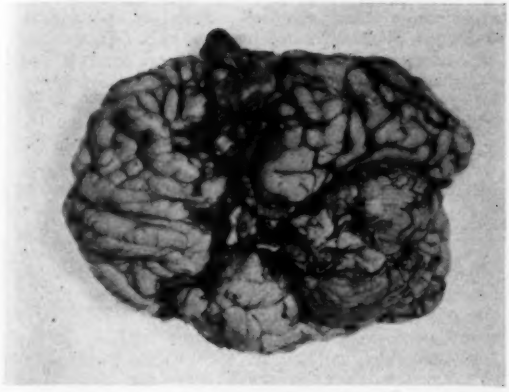

FIG. 8.—Case 2. The brain showing the racemose angioma in the left lateral central sulci.

FIG. 9.—Case 2. Left common carotid artery showing thickening of the intima. Elastica van Gieson, × 11.

FIG. 10.—Case 2. Left parietal lobe. The gyri are shrunken and there are many large engorged blood vessels in the widened sulci. There is focal calcification in the underlying white matter. Thick cresyl violet, × 4.

FIG. 11.—Case 2. Left temporal lobe. Focal intimal thickening with calcification and fragmentation of the elastic lamina. Haematoxylin and eosin, × 100.

A mass of wide convoluted vessels extended throughout the left lateral and central fissures from the base of the brain to the superior sagittal sinus (Fig. 8). The circle of Willis, the internal carotid, and the basilar arteries were dilated. The abnormal vessels drained freely into the superior sagittal sinus and also into the left transverse, left cavernous, and left sphenoparietal sinuses. The cut surface showed that the haemangioma extended down to the insula. The gyri of the opercula and insula were irregularly convoluted and thin. A narrow subpial zone of opaque pale yellow appearance could be traced round the margins of these atrophic gyri and there were small opaque creamy spots and flecks in the centre of the shrunken convolutions. There was marked atrophy of the brain tissue deep to the insula, the distance between the lateral ventricle and the external aspect of the insula being only 1 mm.

Histology.—The liver shows congestion, centrilobular necrosis, and much fatty change in the intermediate zone. The lungs show much alveolar haemorrhage and oedema. The ventricles of the heart are hypertrophied, the right more so than the left. There is fibro-elastic thickening of the intima of both common carotid arteries (Fig. 9) and of the descending aorta. In the pancreas islet tissue is much increased. Some islets are only slightly enlarged, but there are sheets of confluent islets measuring up to 4 mm. across. The islet cells, being grouped about a central capillary, present an ill-defined pseudo-acinar structure; occasionally they appear to be arranged around a central lumen. The exocrine portion of the pancreas is normal. Pathological changes in the brain appear only in the area related to the vascular malformation in the left lateral sulcus. The abnormal vessels lie in the pia arachnoid and do not penetrate the brain substance. They consist of arteries with a well-defined elastic lamina and veins with only thin strands of elastin. These vessels are often of a bizarre configuration (Fig. 10). The arteries show focal fibrous thickening of the intima, sometimes with fragmentation and calcification of the elastic lamina (Fig. 11). Although it is possible to define cortical and medullary zones, the brain tissue shows extensive astrocytic gliosis, containing many vessels and foamy microglia. There are a few small calcific deposits in the white matter.

Case 3.—The patient was a female baby stillborn at 36 weeks' gestation. The mother had a threatened miscarriage at the end of the first trimester.

Necropsy Findings.—The body was that of a grossly malformed female baby with rotation of the trunk through 90 degrees. Much of the intestine and liver were visible within the sac of a large body stalk hernia. The umbilical cord was short (14.5 cm.) and contained only one artery.

The heart was normal in size and had a small high ventricular septal defect. The right renal artery arose from the superior mesenteric artery. The lungs were small and airless and no fissure separated the middle lobe. The intestines were obstructed, due to distortion within the hernial sac. The brain was of normal configuration, but both lateral fissures, the right more than the left, contained prominent, engorged, and convoluted blood vessels. Transection showed atrophy of the right insula and opercula and cystic softening deep to the insula (Fig. 12). There was no abnormality of the brain tissue bounding the left lateral fissure.

Histology.—The right lateral fissure of the brain contains many veins and arteries (Fig. 13). Mostly situated in the pia arachnoid, several penetrate the pial surface and extend into the brain substance (Fig. 14). The vessels are not thrombosed and none shows intimal thickening. The choroid plexus is greatly engorged. The cortex of the insula and frontal and temporal opercula has the highly convoluted appearance of microgyria (Fig. 13). The insula shows much astrocytic gliosis and contains small cysts, fat-filled microglia, and deposits of calcium. In the deep layers of the cortex there are many swollen degenerate nerve cells, some of which are undergoing phagocytosis. The underlying white matter is relatively intact but contains groups of small cells with prominent nuclei and nucleoli which are probably immature nerve cells (Fig. 14). There is slight proliferation of the subependymal glia.

Discussion

In reported series of intracranial tumours, those of vascular origin comprise from 1.4 to 5% (Cushing, 1932; Dandy, 1928; Walshe, 1931). Six are included in 171 cases of intracranial tumours in childhood studied by Bodian (1958). The three cases described in the present paper were necropsied within a few months of each other; but no other instances of intracranial haemangioma have been encountered in this department in the past five years, during which time approximately 1,500 necropsies have been done, mostly on stillbirths and neonates. This suggests that intracranial vascular hamartomas are rare in children.

About one-third of intracranial vascular tumours consist of the type described in the present paper (Cushing, 1932; Russell, 1931). It may be useful to summarize some of the features of 65 such cases reported in the literature (Alpers and Forster, 1945; Arieti and Gray, 1944; Bailey, 1948; Boldrey and Miller, 1949; Buckley, 1928; Byrnes and Boellaard, 1958; Campbell and Ballance, 1922; Craig, 1939; Cushing and Bailey, 1928; Dandy, 1928; Ford, 1952; Jaeger and Forbes, 1946; Lumsden, 1947; Northfield, 1940; Oscherwitz and Davidoff, 1947; Russell and Nevin, 1940; Ward and Horton, 1940; Wolfe and France, 1949; Worster-Drought and Dickson, 1927).

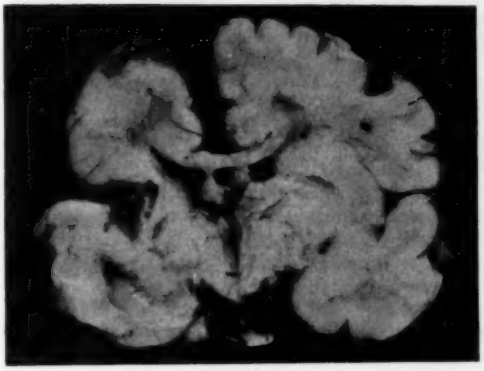

FIG. 12.—Case 3. The brain in coronal section showing atrophy and cystic degeneration of the right insula and an excess of vessels in the right lateral fissure.

FIG. 13.—Case 3. Right lateral fissure. There are many vessels in the fissure and there is microgyria of the insula and opercula. Thick cresyl violet, × 3.

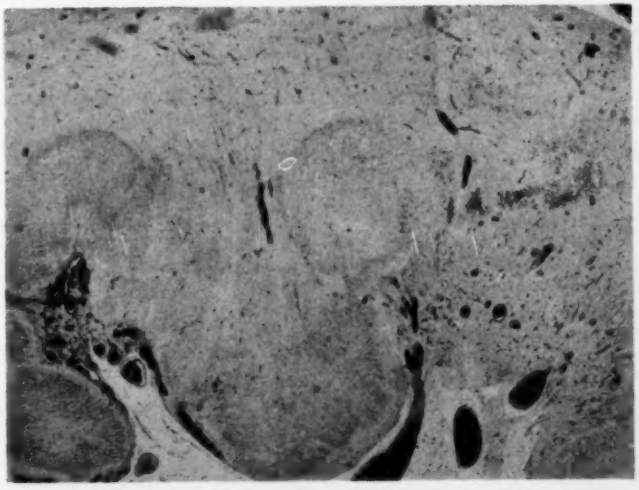

FIG. 14.—Case 3. Right insula showing the abnormal vessels entering the brain substance. The underlying white matter shows focal calcification and ectopic cortical tissue. Thick cresyl violet, × 12.

Just over 70% were male. The onset of symptoms was during the first year of life in about 25%. Cerebral haemorrhage, cerebral palsies, and fits were common manifestations. Port wine angioma in the distribution of the trigeminal nerve and radiological evidence of intracranial calcification, the main features of the Sturge-Weber syndrome, were observed in four of the 65 cases. A direct arteriovenous communication, shown by bruit, pulsation at operation, or post-mortem dissection, was present in about 60% of the cases, but cardiac enlargement has not commonly been recorded. Heart failure was recorded only once, in a boy aged 10 years with an associated angioma of the retina (Northfield, 1940), but the presence of congestive cardiac failure in another two patients described in this paper suggests that heart failure may be commoner than supposed.

A large proportion of the angiomas, about one-third, was paracentral; other parts of the cerebrum were less commonly involved and only three were cerebellar. Extensive lesions may cover the whole of the cerebrum or one cerebral hemisphere. Dandy (1928) reported a case of bilaterally symmetrical venous angiomas in the central sulci, but multiple lesions appear to be distinctly uncommon. The vein of Galen was affected in 10 cases and in nine of these compressed the aqueduct and caused hydrocephalus.

The histological appearances are well described by Arieti and Gray (1944), Buckley (1928), and Cushing and Bailey (1928). The main features of the abnormal vessels are intimal thickening, aneurysmal out-pocketing and splitting of the elastica, sometimes with calcification. The vessels are described as being situated within the pia arachnoid, and penetration of the brain substance as in Case 3 has been reported only once (Byrnes and Boellaard, 1958). Degenerative changes in the related brain substance, observed in all three cases in this paper, are common in detailed histological reports (Arieti and Gray, 1944; Buckley, 1928; Cushing and Bailey, 1928); they consist of extensive gliosis, cystic change, and foci of calcification. Microgyria has not been previously reported in association with these lesions. The gyral pattern in Case 3 was similar to that described by Crome (1952), who noted increased vascularity of related pia arachnoid and brain. It may be of significance that microgyria was present only in Case 3, the only one in which the hamartomatous blood vessels penetrated the brain substance. Abnormal vessels penetrated the brain in the case described by Byrnes and Boellaard (1958) and there was considerable destruction of brain tissue with almost complete obliteration of the gyral pattern; all areas of the cerebral meninges contained abnormal vessels to some extent, but only where these entered the brain were there major alterations of cortical tissue.

Summary

Intracranial racemose angiomas are described in three infants; two acted as arteriovenous fistulas and both infants died of cardiac failure, neither showing clinical evidence of neurological involvement. The third case was stillborn and malformed and there was microgyria near the angioma. The reports of 65 other cases of racemose angiomas have been studied. The lesions are commonest in infancy, tending to affect male children especially. Neurological symptoms predominate and cardiac failure is very rare, although over half have a direct arteriovenous communication. About a third are situated near the central sulcus. Cerebellar sites and multiple lesions are rare. The abnormal vessels are usually situated in the pia arachnoid. Gliosis and calcification occur in the adjacent brain substance. Microgyria has not been previously described in association with racemose angioma.

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PSEUDO TUMOUR OF THE ORBIT AND POLYARTERITIS NODOSA

BY

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Pseudo tumour of the orbit, or chronic granuloma as it is frequently called, is a fairly common clinical condition. Though most cases are of unknown aetiology, a few prove to be manifestations of systemic disease. The purpose of this paper is to report a case where the clinical diagnosis of pseudo tumour of the orbit was made and polyarteritis nodosa, suspected during life, was confirmed at necropsy.

Case Report

W. O. Y., a bus conductor, aged 53 years, was first seen on August 29, 1955. For two months he had had shooting pains in the left temple with frequent headache. A month later the vision of the left eye deteriorated and he was told the eye bulged forwards. He then had two brisk epistaxes. On examination the right eye was normal; the left showed 5 mm. proptosis, limitation of all movements, and absence of the direct light reflex, though the consensual reflex was present. A dense central scotoma was demonstrated on the left side, yet the fundus appeared normal. The pulse was 76 per minute, blood pressure 150/90 mm. Hg. Radiological examination showed opacity of the left posterior ethmoidal region with a suggestion of bone destruction. A blood count was normal apart from slight anaemia (haemoglobin 12.7 g. per 100 ml.), and the plasma proteins showed reversal of the A/G ratio (total 6.6 g. per 100 ml., albumin 2.8 g., globulin 3.8 g.). Blood Wassermann and Kahn reactions were negative. The urine contained a trace of albumin but no abnormal cells and the blood urea was 66 mg. per 100 ml. Exploration of the ethmoidal and sphenoidal air cells revealed thick mucus only: a biopsy of the thickened mucosa showed a granulomatous lesion of chronic, non-specific type with marked endarteritis but no micro-organisms.

Despite treatment with penicillin, he did not improve and within some five weeks was blind in the left eye. The pupil was fixed, the veins were engorged, and optic atrophy had developed. Orbitonometry showed a progressive increase in the proptosis and in the consistency of the orbital contents. Exenteration of the orbit was performed on October 13 (sixth week). A hard pale mass was found in the posterior ethmoidal air cells, penetrating the superior and inferior orbital fissures to surround the optic nerve. The histological report on the biopsy was:

> "The specimen shows a granulomatous lesion described as pseudo tumour of the orbit. As similar material was removed from the ethmoid, it is likely that the lesion originates there. Lesions of the above type have been described in association with periarteritis nodosa. Is there evidence of systemic disease in this patient?" (Dr. R. O. K. Schade).

After the operation the headaches ceased but recurred two weeks later on the right side. Blurring of vision appeared and a dense central scotoma was demonstrated, similar to that which previously developed in the left eye. A course of *x*-ray therapy was given to the posterior orbit in an endeavour to arrest the disease, but the patient's general condition began to worsen; he developed a swinging temperature and became very apathetic. On November 28 signs of peritonitis appeared and at laparotomy a gangrenous, perforated appendix was removed. His condition rapidly deteriorated and he died the following day. There was no terminal rise in blood pressure.

Necropsy

The body was wasted. The left orbit was lined by an apparently healthy graft. Apart from the recent surgical incision in the abdomen there was nothing else of note externally.

Skull.—The dura in the anterior cranial fossae and over the vertex was diffusely thickened and a purulent pachymeningitis was present, localized to these areas. The right orbit, the frontal, maxillary, and anterior and middle ethmoidal sinuses appeared normal. The bone over both the sphenoidal and the left posterior ethmoidal sinuses was elevated and thinned; these air cells contained

a smooth, fleshy mass of yellowish material, homogeneous and adherent to bone. Pus was present in the nasal cavity. The brain, pituitary, and middle ears were normal. The mucosa of the nasopharynx and soft palate was congested; otherwise the mouth, tonsils, fauces, and pharynx were healthy.

Chest.—The lungs were heavy and on section showed areas of early bronchopneumonia: the bronchial tree and main pulmonary vessels were normal. The heart was of normal size. The coronary arteries, especially the descending branch of the left vessel, were tortuous and showed many nodular thickenings which on section were small aneurysms, filled with recent thrombus. The aorta and its main branches were normal apart from atheroma.

Abdomen.—A recent fibrinous peritonitis was present. Numerous mucosal erosions were present in the bowel, especially in the ileum and rectosigmoid. The liver was of normal size and the parenchyma was mottled and congested: numerous ante-mortem thrombi were present in major branches of the portal vein and in nodosities of the hepatic artery (Fig. 1). The spleen weighed 300 g. and was extensively infarcted (Fig. 2). The kidneys together weighed 520 g. Multiple recent infarcts were present in each and the cortices were extensively swollen and blurred. Many of the main branches of each renal artery contained thrombus (Fig. 3). Infarcts were present in each testis (Fig. 4).

The gastroduodenal, left spermatic, and left gastric arteries and major branches of the splenic, hepatic, renal, and superior mesenteric arteries were grossly nodular, the aneurysms all containing ante-mortem thrombus. The other main vessels were healthy. All other organs and tissues were normal. Bacteriological examination of material from the paranasal sinuses was negative.

Histology

Review of the biopsies from the left orbit and ethmoidal sinuses shows in each a chronic granuloma of non-specific type. In the orbit the granuloma tightly ensheaths the optic nerve and has replaced much of the orbital fat. The dense cellular infiltrate consists largely of macrophages, plasma cells, lymphocytes, and neutrophil polymorphs: eosinophil polymorphs are quite frequent, but there is no epithelioid cell reaction and the infrequent giant cells are of foreign body type. The infiltrate, perivascular in places (Fig. 5), has in most areas fused to form a solid mass. The lymphocytes are in places arranged into pseudo follicles without germ centres. At the periphery, the lesion invades the orbital muscles. Many vessels, both arterioles and venules, show perivascular infiltration but are otherwise normal.

The ethmoidal mucosa is greatly thickened by fibrous tissue and patchily infiltrated by cells of similar type but fewer in number than in the orbital granuloma. There are no giant cells and no focal granulomata. The arteries show fibrosis of all layers with reduplication of elastica and narrowing of the lumen. No defects of elastica are present, and the lesions, though reminiscent, are not diagnostic of polyarteritis nodosa. Similar changes are seen in the necropsy specimens from the sphenoidal and left ethmoidal air cells. No micro-organisms are seen in sections from each granuloma stained by the Ziehl-Neelsen, Gram, and P.A.S. methods. Areas of myelin degeneration are present in the right optic and oculomotor nerves; there is infarction in the ocular muscle, but no granuloma is present on this side.

The necropsy material shows vascular lesions (Figs. 6–11) in many organs and tissues (Table I).

TABLE I
SITES OF LESIONS

Granuloma	
Left orbit	
,, ethmoidal sinuses	
Both sphenoidal ,,	
Arteritis	
Recent	*Old*
Coronary, gastroduodenal, left gastric and left spermatic arteries	Right optic nerve
Branches of splenic, hepatic, renal, and mesenteric arteries	Right ethmoidal sinuses
Right optic nerve	Nasal septum, tongue, vocal cord, thyroid, liver, pancreas, kidney, spermatic cord, seminal vesicles
Right orbital fat	Brachial plexus
Dura mater	Sciatic nerve
Epiglottis, trachea, stomach, ileum, rectum, liver, pancreas, spleen, kidney, epididymis	
Infarction	
Heart	
Spleen	
Pancreas	
Kidney	
Testes	
Right ocular muscles	
Acute inflammation	
Pachymeningitis	
Tracheitis, bronchitis, and bronchopneumonia	
Proctitis	

Small and medium-sized arteries are principally involved though occasional affected arterioles and even capillaries are seen in the spleen and kidneys. Veins are not affected though thrombi are present in small veins in the liver, kidney, and lacrimal glands. The lesions are focal and often segmental

FIG. 1.—Liver slice showing numerous ante-mortem thrombi in branches of portal vein and hepatic artery. Irregular fixation has increased the mottling of the tissue.

FIG. 2.—Cut surface of spleen to show the extensive infarction.

FIG. 3.—Slice of left kidney showing multiple recent infarcts. Ante-mortem thrombus is present in many branches of the renal artery.

FIG. 4.—Hemisection of right testis to show the extensive infarction.

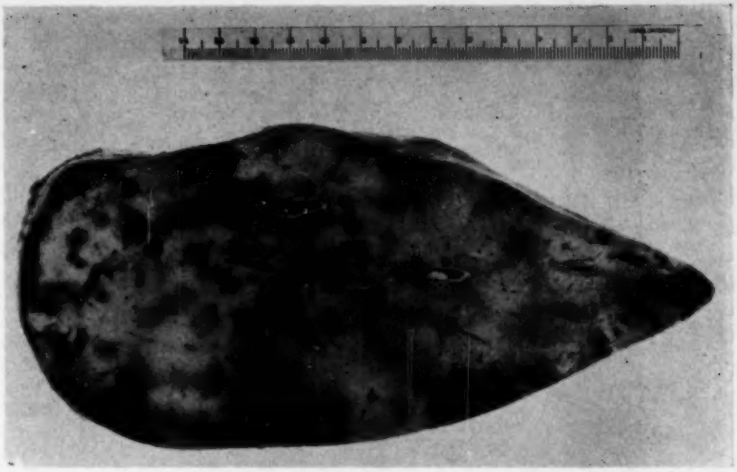

FIG. 1

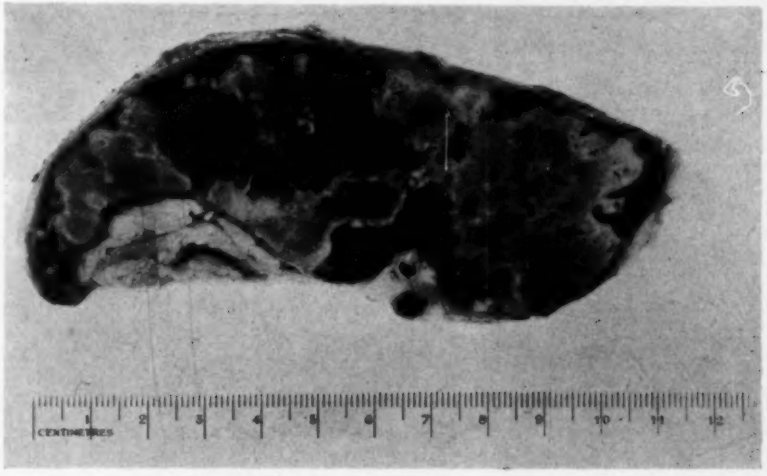

FIG. 2

FIG. 3

FIG. 4

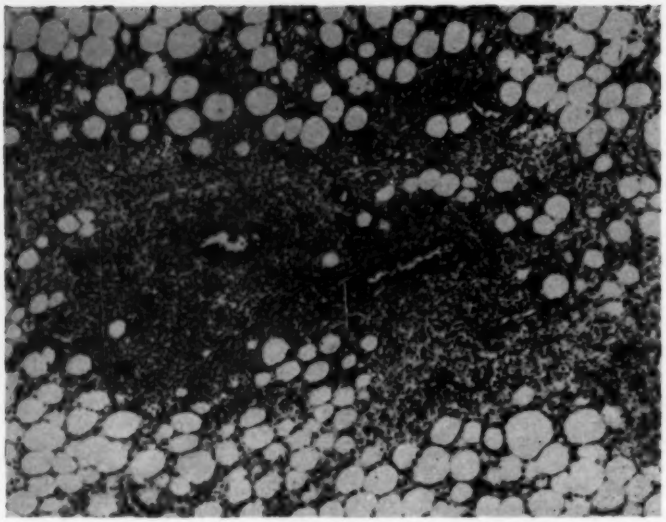

FIG. 5

FIG. 5.—Low-power view of part of the orbital granuloma showing that in parts the infiltrate is perivascular. Haematoxylin and eosin, × 70.

FIG. 6.—Section of a coronary artery with acute necrosis of a segment of the wall and almost complete occlusion of the lumen by thrombus. Serial sections of the lesion confirm that the vessel is slightly dilated at this point. Haematoxylin and eosin, × 27.

FIG. 7.—This section of pancreas shows an acute lesion at a point of arteriolar branching. One limb contains thrombus. Haematoxylin and eosin, × 112.

FIG. 6

FIG. 7

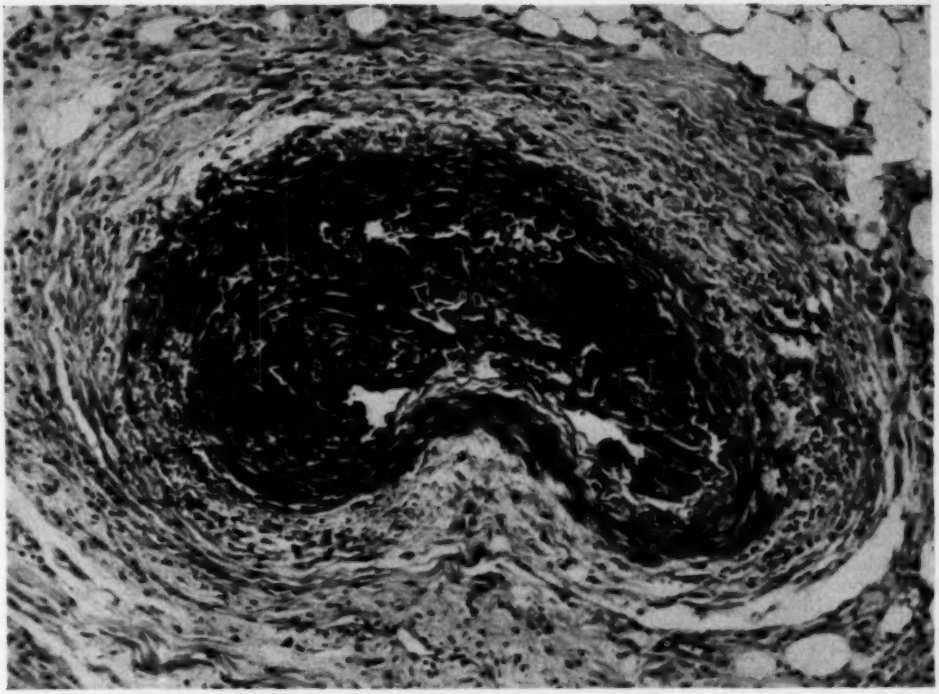

FIG. 8.—Acute necrosis and thrombosis of an arteriole in the adipose tissue of the right orbit. Haematoxylin and eosin, × 144.

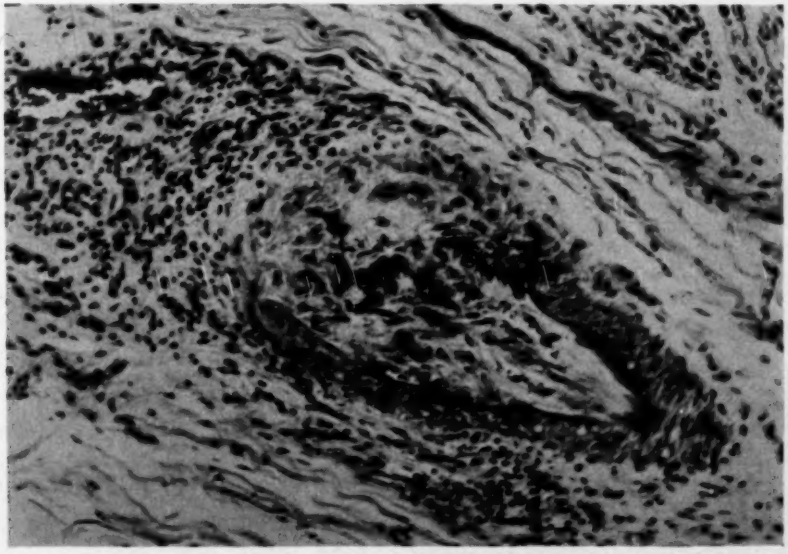

FIG. 9.—Healing lesion in an arteriole from the right superior oblique muscle. Part of the vessel wall has been replaced by granulation tissue and the lumen is occluded by partly organized thrombus. Haematoxylin and eosin, × 160.

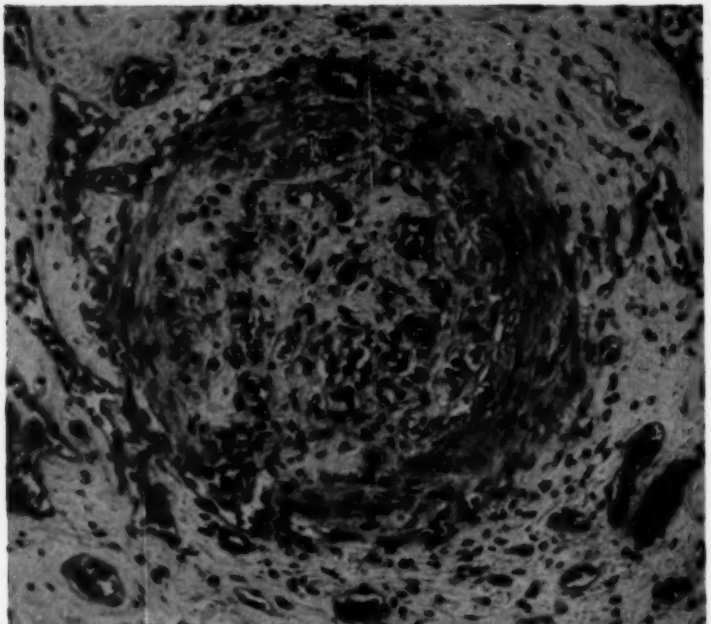

FIG. 10.—This section of a nerve trunk from the brachial plexus shows a healing lesion in an arteriole. The wall of the vessel is patchily scarred and the lumen is filled with fibrous tissue containing numerous capillaries. Haematoxylin and eosin, × 185.

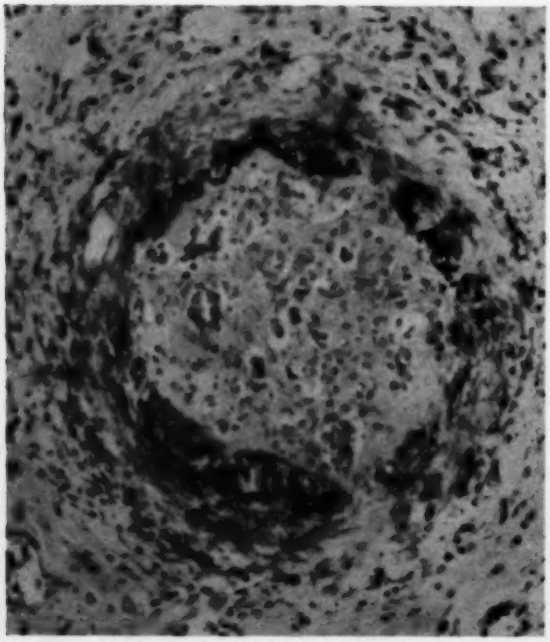

FIG. 11.—Serial section to Fig. 10, to show fragmentation of the elastica. Weigert's elastica and scarba red, × 150.

and appear to have a predilection for sites of branching (Fig. 7). In the acute phase (Figs. 6–8) the affected vessels show material, giving the staining reactions of fibrin, seeping into, and sometimes beyond, their walls. Oedema, especially of the intima, infiltration of all layers with neutrophil polymorphs and macrophages, and thrombosis, are other features. In places, the wall of the larger affected vessels has dilated to give a small aneurysm (Fig. 6). Healing lesions (Figs. 9–11) show organization of and formation of new vessels in the luminal thrombus whilst the hallmark of the healed lesion is segmental absence of the elastica (Fig. 11). These lesions are typical of polyarteritis nodosa. Associated with the vascular disease are infarcts of heart, spleen, pancreas, kidneys, testes, and the right ocular muscles.

No vascular or granulomatous lesions are present in the trachea, lung, or bronchi, and no giant-cell granulomata are seen in any of the visceral sections. A few glomeruli show fibrinoid change of capillaries, but most are normal. The aorta, subclavian, iliac, and carotid arteries are normal, apart from atheroma. Non-specific inflammatory lesions include an acute pachymeningitis, acute tracheitis, bronchopneumonia, acute proctitis, and acute on chronic rhinitis and antral sinusitis.

Other organs and tissues examined, including brain, skin, voluntary muscle, tonsil, salivary gland, lymph nodes, and bone marrow, appear normal.

Discussion

This case presents the coexistence of two syndromes, pseudo tumour of the orbit and polyarteritis nodosa, both incompletely understood and both uncommon. The association is so rare that I have found only one detailed report, that by Hope-Robertson (1956). Two further cases are briefly discussed by Reese (1951, 1956) whilst Case 1 of Howells (1955) and that of Bellomio (1953) are probably further examples. It is not mentioned by Duke-Elder (1952) in his section on chronic orbital inflammation nor by Jackson (1958) in his recent review of pseudo tumour.

The term "pseudo tumour of the orbit" has a clinical rather than a pathological connotation and "may be taken as embracing a considerable group of cases which are difficult to differentiate clinically from an orbital tumour but which on pathological investigation or by their resolution under treatment are shown to be granulomata of chronic inflammatory type" (Duke-Elder, 1952). Reese (1951) has distinguished five varieties, with the following histological features: (1) Prominent lymph follicles; (2) focal and perivascular infiltrations of lymphocytes and involvement of muscle; (3) chronic inflammation widely spread throughout the tissue and varying degrees of fibrosis and polymorph infiltration; (4) early fibrosis with little inflammation; (5) inflammation in a pre-existing angioma.

The lesion in the present case, in which there is both involvement of muscle and the development of occasional lymph follicles, does not fall readily into any of these categories; it most closely resembles type 3.

Occasionally, orbital granulomata are associated with systemic disease, and Reese (1951) has noted the association with sarcoidosis, leukaemia, syphilis, tuberculosis, and Hand-Schüller-Christian disease. Excluding these cases, and those of type 5 in which an angioma is probably the original lesion, the aetiology of pseudo tumour is unknown.

Though there is widespread agreement that polyarteritis nodosa is not a homogeneous entity, no commonly agreed classification exists. Perhaps the best is that of Rose and Spencer (1957), who differentiated between cases with and without pulmonary involvement. In the former there is usually a prior respiratory illness such as asthma or pneumonia; eosinophilia is common; necrotizing parenchymal and vascular lesions are found in the lungs; and the affected vessels, usually small, often show giant-cell granulomata. This group appears to include several of Zeek's (1952) categories, especially "allergic granulomatous angiitis," and a history of hyperergic disease is common to most. In the second group, granulomatous lesions are rare and the pulmonary vessels are not involved while there is no association with respiratory illness. This group is apparently identical with the "classical" polyarteritis nodosa of older authors and the "true periarteritis nodosa" of Zeek. Though hypertension has often been postulated as the causal factor in this type, Rose and Spencer (1957) have shown that it is confined to those cases in which there are healed vascular lesions in the kidneys; it would appear therefore to be a sequel rather than a cause of the disease. In the present case, the changes were clearly of this second category; there were few healing lesions in the kidneys and hypertension did not develop.

The interest in this case lies in the possibility that the coexistence of the two lesions, pseudo tumour of the orbit and polyarteritis nodosa, may lead us to a clearer understanding of the aetiology of each. Three possibilities exist: first, that the polyarteritis was secondary, both in time and in causation, to the orbital granuloma; secondly, that the granuloma was a consequence of the polyarteritis; and, thirdly, that the presence of the two lesions was coincidental. Before considering these propositions, we may note that there are two sets of circumstances in which granulomata and polyarteritis nodosa are constantly associated. In one, "allergic granulomatous angiitis" (Churg and Strauss, 1951), asthma is a common factor and allergy the probable cause. In the other, to which the eponym "Wegener's granulomatosis" has been applied, there is granulomatous ulceration of the upper air passages. I have shown elsewhere (Walton, 1958) that the polyarteritis in this group is secondary to the granuloma and is probably the result of a hypersensitivity reaction.

To return to the three possibilities, asthma was not a feature of the present case, but it is tempting to suggest that, by analogy with Wegener's granulomatosis, the polyarteritis in it was likewise secondary to a granuloma, in this case arising in the orbit and sinuses. There are, however, significant differences between Wegener's granulomatosis and this case. In the former condition the granulomata have giant cells and central necrosis and are widely distributed. The vascular lesions are akin to those in Rose and

Spencer's (1957) first group, small vessels in the lung being especially involved. In the case under discussion, no giant cells were found in the lesions, the granulomata were present only in the orbit and paranasal sinuses, the lungs were not affected, and the vascular lesions, involving mainly vessels of medium size, clearly come into Rose and Spencer's second category. Furthermore, no clinical evidence of hypersensitivity was noted. There is thus no convincing evidence that the vascular lesions were caused by the orbital granuloma.

More likely is the second possibility that the orbital and sinusoidal lesions were a result of the polyarteritis, being the sequel of confluence of numerous perivascular infiltrates. In the case of polyarteritis nodosa with chronic orbital myositis described by Bellomio (1953), inflammatory lesions of the orbital muscles were thought by the author to be the direct result of polyarteritis. In Hope-Robertson's (1956) case an interval of two and a half years elapsed between the diagnosis of pseudo tumour of the orbit and the demonstration at necropsy of polyarteritic lesions. In the present case, re-examination of serial sections from the orbital and sinusoidal biopsies showed no vascular lesions of the kind at necropsy. This proposition is therefore no more than surmise and very possibly the coexistence of the two lesions is mere coincidence. Nevertheless, it seems justifiable to suggest, on the basis of this case, that in cases of orbital granuloma, especially in the presence of symptoms and signs of widespread disease, a vasculitis should be considered as a possible aetiological factor.

Summary

A case is reported in which the presenting clinical picture was that of pseudo tumour of the orbit. At necropsy, polyarteritis nodosa, suspected during life because of the perivascular nature of the orbital granuloma, was confirmed. The bearing this rare association has on the aetiology of pseudo tumour, the relationship with Wegener's granulomatosis, and the classification and aetiology of necrotizing arteritis are briefly discussed.

I am indebted to Mr. H. Vernon Ingram for permission to publish this case, to Mr. A. E. Young and Mr. J. W. Corkhill for the illustrations, and to Professor J. B. Duguid, Professor A. C. Lendrum, Dr. R. O. K. Schade, Dr. I. Rannie, and Dr. W. W. Park for advice and encouragement.

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MICROGYRIA AND CYTOMEGALIC INCLUSION DISEASE IN INFANCY

BY

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Many different malformations of the central nervous system can now be produced in the offspring of animals exposed during pregnancy to *x* rays, drugs, and dietetic deficiency or excess of vitamins, but proof of environmental causation of analogous human conditions is understandably scarce. Occasional neural, somatic, or gonadal maldevelopment has been reported after the administration to pregnant women of aminopterin (Thiersch, 1956), thiouracil (Morris, 1953), methyltestosterone (Nellhaus, 1958), and progesterone (Reilly, Hinman, Pickering, and Crane, 1958; Wilkins and Jones, 1958). Nevertheless, maternal rubella and ionizing radiation remain the only widely known extrinsic causes of human embryopathy. Hence the importance of the suggestion that another, probably infective, condition, cytomegalic inclusion disease, may be also responsible for neural malformation (Diezel, 1954).

It has been known for some time that the brain is one of the organs which may display the characteristic inclusions of cytomegalic inclusion disease. Haymaker, Girdany, Stephens, Lillie, and Fetterman (1954) collected seven such cases in the literature and added one of their own. In addition to the inclusions, some of these brains showed hydrocephalus, periventricular calcification, focal softening, haemorrhages, astrocytic proliferation, perivascular inflammatory infiltration, and ependymal granulations. These are, of course, evidence of neural disease, but not necessarily of malformation. Diezel was the first to report the association of cytomegalic inclusion disease with microgyria, or micropolygyria. Microgyria is a true cerebral malformation characterized by an excess of small abnormally formed gyri (Crome, 1952). Histological and embryological considerations suggest that the cause of this condition operates before the sixth month of foetal development.

The patient described by Diezel was an infant weighing 2,500 g. at birth who developed neonatal jaundice, ascites with hepatomegaly, and splenomegaly, and died at 24 days. Inclusion bodies were found in the kidneys, liver, and brain. The brain weighed 130 g. and showed microgyria of the neocortical areas. Necrosis was present in the medial basal regions of both temporal lobes and in the cerebellum. The optic nerves were atrophic, there was marked hydrocephalus, and the corpus callosum was thin. On microscopy, the necrotic areas showed infiltration by lymphocytic and plasma cells with many compound granular corpuscles, macrophages, and calcified particles. The olfactory nerves were the seat of marked inflammatory change. Typical inclusions of cytomegalic inclusion disease were seen in the leptomeninges, ependymal, and glial cells, as well as in some of the undifferentiated matrix cells arranged in numerous dense periventricular foci. The only mature neurons to show inclusions were a few Purkinje cells, found after prolonged search. Architectonically, the palaeocortex and archicortex were normal and the relatively older occipital cortex was better developed than the rest of the neocortex. It appeared therefore to the author that the virus of cytomegalic inclusion disease attacked the foetus at a time when migration of nerve cells from the periventricular matrix to the periphery was complete in the older, and still afoot in the newer parts of the brain, i.e., the beginning of the fourth month of gestation.

Diezel was able to re-examine histological preparations from the brains of the two cases of cytomegalic inclusion disease previously reported by Hartmann (1948) and by Haymaker and his co-workers. He found microgyria in one of these, whilst the other had an anomaly of gyri, possibly of microgyric nature. Microgyria was possibly present in a case of cytomegalic inclusion disease presented by Mercer, Luse, and Guyton (1953); the brain of this child had an irregular, thin-walled cyst, 1.5 cm. in diameter, in the right parietal lobe, the cortical surface around the cyst was slightly

depressed and firm, while the irregular narrow gyri in this area had a "wrinkled" surface. A further definite example of cytomegalic inclusion disease with microgyria was recently demonstrated in this country by Strich (1958, personal communication), and it is the object of this communication to record another case.

Case Report

This girl was the issue of the second pregnancy of a 25-year-old unmarried mother. Two years previously, she had had a miscarriage at 3 months, followed a few months later by possible glandular fever with lymphadenopathy and a rash. The Paul–Bunnell test was not done. Apart from slight vaginal bleeding at the 12th week of gestation, her second pregnancy was normal and the labour began spontaneously at 32 weeks. Labour was complicated by mild ante-partum haemorrhage and resulted in delivery by the vertex of a female infant weighing 2,020 g. The placenta (760 g.) was large and friable.

At birth, the baby showed numerous skin haemorrhages of face, trunk, and lower limbs. The abdomen was huge and lax with considerable enlargement of the liver and spleen. Respirations were moaning and grunting and there was much rib recession. The submaxillary salivary glands were not enlarged. In spite of oxygen therapy and treatment with penicillin and streptomycin, her condition rapidly deteriorated; moaning respirations continued and occasional convulsive movements preceded death at 31 hours.

Investigations

Mother.—The blood group was O, Rh negative (to anti-D). The serum contained no atypical antibodies, and anti-A and anti-B titres were within normal limits.

Baby.—The blood group was B, Rh positive (to anti-D). The direct Coombs test was negative. Serum bilirubin (at birth) was 2.3 mg. per 100 ml. Capillary blood contained 10.36 g. haemoglobin per 100 ml. (Haldane). Colour index was 1.32; R.B.C.s 2.64m. per c.mm.; total nucleated cells, 12,700 per c.mm.; platelets, 142,000 per c.mm.

Pathological Findings

The body weighed 1,910 g., and was 43.5 cm. (crown–heel) long. The head was microcephalic, the circumference measuring 27 cm. compared with 34.7 cm. which is the mean for this age. The abdominal circumference was 37 cm. There was generalized mild oedema and slight jaundice, with numerous haemorrhages into the skin, conjunctivae, and umbilical cord.

The heart showed marked dilatation of the right side, and patency of the ductus arteriosus. The lungs (R. 13 g., L. 11 g.) were pale purple and poorly aerated. The spleen weighed 14 g. (average normal for age, 6 g.) and showed patchy capsular thickening. The liver weighed 112 g. (average normal for age, 88 g.). It was light green and had a few small subcapsular groups of cysts 1–2 mm. in diameter. The gall-bladder contained colourless mucoid material. The peritoneal cavity contained about 200 ml. of clear yellow fluid. The stomach showed a small mucosal erosion. The small intestine contained meconium and was bound together by fine fibrous adhesions. The large intestine was collapsed, containing only pale green mucus. The thymus weighed 5 g. and the left femur was normal. The occipital bone was firmly united with both parietal bones and showed a prominent posterior projection. There were small haemorrhages in the dura.

Histologically, the lungs showed extensive aspiration of amniotic fluid and areas of well-marked hyaline membrane formation with occasional alveolar haemorrhages. A single large cell was seen in one of the sections which was attached to the alveolar lining and contained a large oval intranuclear inclusion separated by a clear halo from the nuclear membrane showing condensation of chromatin. Its cytoplasm contained many small basophilic inclusions.

The spleen showed diffuse generalized haemopoiesis. Many haemopoietic foci were also present in the liver, which showed a considerable accumulation of haemosiderin in the parenchymal and Kupffer cells. The subcapsular cysts were lined by fibrillary material and were separated from the capsule by a thin zone of liver cells. A few degenerate inclusion cells were seen in the bile duct of one portal tract.

Both kidneys showed cortical foci of interstitial inflammatory cell infiltration mainly by lymphocytes and plasma cells. Dilated tubules containing many typical inclusion cells were present in some of these inflammatory foci (Fig. 1). Glomeruli were mostly normal, but some were hyalinized and one contained an inclusion cell in a glomerular tuft. There was a moderate amount of haemorrhage into the medulla and excessive haemopoiesis. Many typical inclusion cells without accompanying inflammation were present in the submaxillary salivary gland. The parotid glands were normal.

Central Nervous System.—The unfixed brain weighed 87 g., the range for a baby of 2,000 g. being about 250 to 350 g. from our unpublished observations.

The brain, brain-stem, and cerebellum had been initially cut sagittally through the midline and,

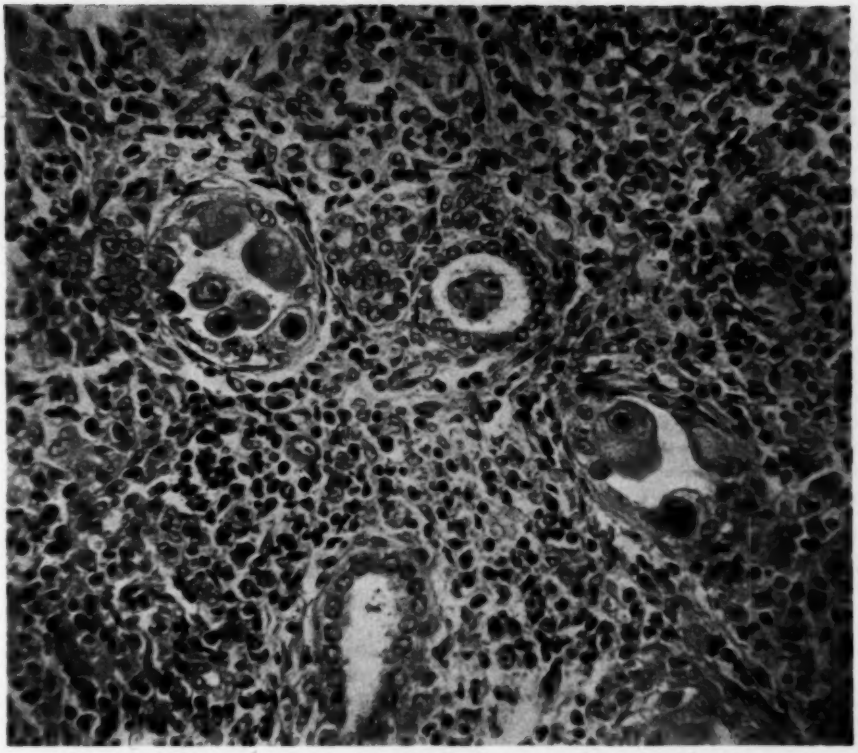

FIG. 1.—Kidney, showing dilated tubules with typical inclusion cells and interstitial inflammatory cell exudate. Haematoxylin and eosin × 350.

FIG. 2.—External aspect of right cerebral hemisphere.

FIG. 3.—Medial aspect of right cerebral hemisphere.

when re-examined for purposes of the present study five years after death, the material was in two parts. The left side of the brain had been sectioned coronally and partly embedded in paraffin. Additional material was now embedded in celloidin, further sections cut of the paraffin-embedded material, and frozen sections used for fat staining and for silver impregnation methods. The usual general and neurohistological methods were employed. The complete right half of the brain had been stored as a museum specimen in Kaiserling's solution, and, as little was to be gained by sectioning it, only the macroscopic appearances will be presented below.

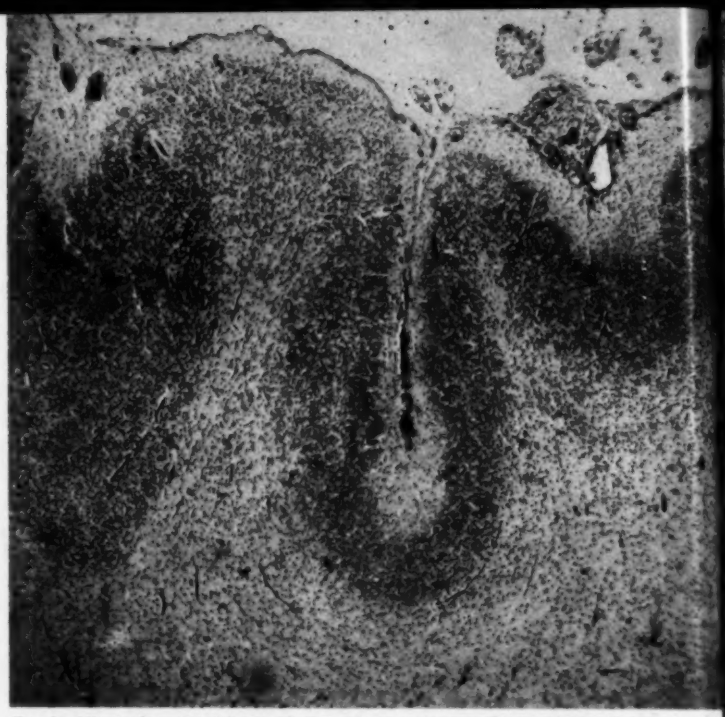

FIG. 4.—Microgyric cortex with area of nerve cell loss at the crown of one of the gyri. Cresyl violet

The complete right half weighed 31 g. after fixation, and measured $6.5 \times 3.8 \times 2.5$ cm. The meninges were blood-stained, particularly over the cerebellum. A large, oval, depressed area, $3.0 \times 1.1 \times 1.0$ cm., covered by a single-layered meningeal membrane, was situated in the region of the Sylvian fissure. When the meninges were removed, a porencephalic defect came into view (Fig. 2). It extended from the surface into the dilated lateral ventricle, its inner wall being formed by the internal aspect of the medial part of the hemisphere, and the floor by the rounded elevations of the basal ganglia bearing posteriorly some tufts of the choroid plexus. Microgyric verrucosity was widespread over the entire surface of the brain, being perhaps more marked in the frontal lobe than elsewhere. The thickest and apparently most mature portion of the brain was the temporal lobe, and this showed least microgyria. An elongated, pale, hard, probably calcified segment of tissue, 1.2×0.2 cm., extended antero-posteriorly in the inferior

FIG. 5.—Round and columnar foci of primitive matrix cells. Cresyl violet × 30.

FIG. 6.—An area of softening breaking into the ventricle. Cresyl violet × 30.

discoloration, was present on the medial surface above the corpus callosum. The lateral and third ventricles were dilated. The best-formed part was, as in the right hemisphere, the temporal lobe. No calcification could be detected on naked-eye examination.

The main features elicited by the histological examination were cyto-architectonic details of the micro-gyria and the presence of focal cerebral necrosis.

The microgyric cortex was formed in most areas by a fairly uniform molecular layer and a deeper cellular layer of round neurones with no evidence of further lamination (Fig. 4). Most of the sulci were shallow, some being scarcely indicated on the surface, and the neuronal layer between and beneath them was convoluted accordingly, presenting either gentle or more acute

temporal gyrus close to its junction with the fusiform, and the surface of the brain around it had a brownish tinge. With the exception of the temporal lobe, gyri on the external aspect of the hemisphere bore no recognizable relation to the normal pattern. The medial aspect of the hemisphere was better developed (Fig. 3). The cingulate gyrus was relatively well formed, and the calcarine and parieto-occipital sulci were identifiable and in normal position. Microgyria were, however, superimposed upon the basic gyri. The corpus callosum was minute, 1.2 cm. in length and only a fraction of a millimetre in thickness. The fornix was very thin, but the foramen of Monro was identifiable. Only the optic, third and fifth cranial nerves, all very small, were recognizable. The brain-stem and the cerebellum were relatively large and normal. The spinal cord was thin but otherwise normal.

When the left hemisphere was partially reconstituted from the remaining blocks preserved in formalin, it proved generally similar to the right in size and shape, but there was no porencephaly on the lateral surface. A large deficiency (5.3 × 0.8 cm.), covered by a layer of meninges, and bordered by areas of rusty-red

FIG. 7.—Mening+-cortical plaque. Haematoxylin and eosin × 100.

undulations, and bearing in places rounded or square extensions directed towards the surface of the brain. Where gyri were uncommonly numerous, they were sometimes cut tangentially and parts of a second neuronal layer would then appear deep to the external one. The above basic pattern was often modified. Focal deficiencies of neurons were present in the neuronal layer. A few rarer areas showed also a more "classical" type of microgyric lamination consisting of four layers: (1) molecular, (2) densely cellular neuronal, (3) sparsely cellular, and (4) deep neuronal merging with the subjoining "white matter." Some axon cylinders were present in the molecular and third layers, which contained, however, no trace of myelin sheaths. Lastly, the more mature cortical areas in the temporal and, to a lesser extent, occipital lobes had broader and thicker gyri with deeper sulci, and showed also some indication of normal stratification and neuronal differentiation.

The centrum semiovale was formed by a dense reticulum of fine fibres and a moderate number of glial cells. Many of these cells could not be classified with any certainty; some were unipolar spongioblasts, others more mature astrocytes and oligodendrocytes. Axon cylinders were numerous but there was no evidence of myelination. Many dense round and columnar foci of undifferentiated matrix cells were situated in the "white matter" (Fig. 5), chiefly around, and some distance from, the ventricles.

The ventricles were lined by tall columnar ciliated epithelium, but this was largely denuded. Some ependymal granulations were also present, and in a few areas the ependyma had grown inward into the periventricular tissue, either diffusely or in the form of single or multiple tubules. Haemosiderin was present upon the surface of the choroid plexus.

The necrotic areas varied in size and structure. Some were situated in the cortex, others periventricularly, while at least two had traversed the entire thickness of the hemisphere forming a narrow, irregular, cleft-like communication between the surface and the ventricles. The seemingly fresh lesions showed rarefaction or complete softening and contained many fat-laden phagocytes with some anisotropic intra- and extracellular debris, particles of calcium, and haemosiderin granules. Where the softened tissue had broken into the ventricles, the gap was encircled by a collar of thickened ependymal tissue (Fig. 6). Other, presumably older, foci of necrosis showed less phagocytosis and cellular proliferation and more calcification. These foci were mainly periventricular, the largest being situated in the centre of the superior medullary velum and measuring some 3 mm. in diameter. In addition to the necrosis, there were also many small fresh haemorrhages in the meninges and brain, and some of the intracerebral blood vessels were cuffed by phagocytic fat-containing cells.

The meninges were normal over most of the cortex, but above and around areas of cortical necrosis they were thickened and fused with the subjoining tissue forming flat or wedge-shaped plaques (Fig. 7). These consisted of meningeal and glial cells with an increased amount of collagen and reticulin. Blood vessels in these plaques were dilated and often plexiform. Many macrophages containing neural fat with calcium and haemosiderin granules were present in the interstices of the tissue.

Where examined, the basal ganglia seemed better developed and preserved than the cortex, but no detailed anatomical study was attempted. The cerebellum was normal, although the ependyma over the fourth ventricle showed some "granulations." The brain-stem and spinal cord also seemed normal for the stage of development showing some myelination of fibre tracts.

Cytomegalic inclusions were searched for in many sections stained by the usual methods, including Lendrum's phloxine tartrazine. No certain inclusions were found, but a few cells among the phagocytes in the necrotic areas were, perhaps, equivocal. Their cytoplasm contained irregular or rounded, more or less eosinophilic bodies, surrounded by a clear halo. These could be interpreted as modified inclusions, but it seemed more reasonable to regard them as ingested particles. Intranuclear inclusions were certainly absent in the many sections examined.

Discussion

The clinical features of the present case—neonatal jaundice, ascites, hepatomegaly, splenomegaly, anaemia, erythroblastaemia, and widespread haemorrhages—were those usually found in neonatal generalized cytomegalic inclusion disease (France, 1951), and also resembled closely the picture in the previously described cases with microgyria. The pathological features in the central nervous system showed certain differences. Inclusions were not found in the present case and calcification was not as gross as in the previous instances. Porencephaly was absent in the earlier cases, which showed, however, like the present one,

focal necrotic change. The temporal and the occipital lobes were better developed in both the present and in Diezel's case than the other parts of the cortex, and microcephaly was also a common feature. Despite the differences, the clinical and morphological similarity seems remarkable and strongly suggests that the cases are instances of the same disease.

Viewed as microgyria, the present case is somewhat anomalous. The abnormal pallium in older individuals with this condition is usually characterized by easily recognizable and widespread four-layer lamination. The marginal layer contains few nerve cells. The second layer is densely cellular, while the third is sparsely cellular, containing many myelinated nerve fibres which extend at intervals as stout radial bundles into the core of the overlying microgyric convolutions. The deepest, fourth layer contains again nerve cells. This arrangement was modified in the present case. The typical four-layer lamination was rare, while the first and third layers contained no myelin. These differences are probably accounted for by the immaturity of the present brain. No myelin was in fact present in any part of the centrum semiovale, and the numerous islands of undifferentiated matrix cells indicated incomplete migration of neuroblasts to the periphery. It is likely that, had the child lived, continued maturation would have resulted in a more typical picture.

While the occasional association of cytomegalic inclusion disease with microgyria is thus established, it is impossible to accept unreservedly Diezel's contention that the former is a virus infection and that the association of the two is sufficient evidence of the infective origin of microgyria in such cases.

Current views on the nature of cytomegalic inclusion disease may be summarized as follows. Although the inclusions of cytomegalic inclusion disease have been reported in the salivary glands of 10% to 30% of infants examined by routine necropsy, the generalized disease is far less common, somewhat more than 100 necropsy cases having so far appeared in the literature. Only two fatal cases occurred in the neonatal period at the Mothers' Hospital during 1948 to 1959 of a total of 20,220 infants delivered.

There is strong evidence in favour of human cytomegalic inclusion disease being due to a species-specific salivary gland virus comparable to that isolated from mice (Smith, 1954). Later, Smith (1956) recovered viruses from two human cases producing cytopathological change in cultures of human fibroblasts like those which are pathognomonic of cytomegalic inclusion disease. These results were obtained from the salivary gland of an infant showing typical inclusion cells histologically, and from the kidney of an infant dying with the generalized disease. Both viruses were neutralized by the serum of infants dying of cytomegalic inclusion disease and by the mother of an affected infant. Similar viruses have been isolated from spontaneously degenerating tissue cultures of human adenoids (Rowe, Hartley, Waterman, Turner, and Huebner, 1956), and from infants with illnesses resembling cytomegalic inclusion disease (Weller, Macauley, Craig, and Wirth, 1957).

Even if cytomegalic inclusion disease is a virus disease, its occasional association with microgyria may be fortuitous: a foetus with a neural malformation may be infected later. It is therefore important to continue reporting such cases: the causal relationship between the two would be strengthened by larger numbers, especially if they remain as similar to each other, clinically and pathologically, as they have been hitherto.

Whatever the trend of future observations, it is certain that cases of microgyria without any evidence of cytomegalic inclusion are common. Thus, of 282 brains of individuals with encephalopathy or mental retardation examined at the Fountain Hospital, 16, including the present one, showed microgyria. Four of the 16 showed no cytomegalic inclusions in the brain or elsewhere; examination of the others is still incomplete. Hence cytomegalic inclusion disease could be only one of several causes of microgyria, and this is not surprising, since it is well known from experimental work that the same malformation may be produced in different ways. Thus, a condition closely resembling human microgyria and pachygyria, another cerebral malformation having many features in common with microgyria (Crome, 1956), was, for example, successfully reproduced in rats (Riggs, McGrath, and Schwarz, 1956) by exposing pregnant animals to irradiation five to nine days before confinement. Human pachygyria after maternal irradiation by *x* rays during pregnancy had been previously reported by Uiberrak (1942), and a case of microgyria in a child born to a mother after an attempted suicide with carbon monoxide in the fifth month of the pregnancy by Hallervorden (1949).

Summary

Several instances of microgyria associated with cytomegalic inclusion disease have been observed

by previous authors. A further example is presented in this communication and the significance of the association discussed.

Our thanks are due to Dr. H. M. M. Mackay for permission to publish the clinical details of this case.

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TRACHEOPATHIA OSTEOPLASTICA

BY

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Tracheopathia osteoplastica is a rare benign abnormality of the trachea and bronchi characterized by numerous cartilaginous and bony nodules within the tissues of the submucosa without ulceration of the surface.

The trachea, and, to a lesser extent, the bronchi, become narrow, rigid, and inelastic, the surface of the air passage presenting a corrugated and "cobblestone" appearance due to the projection of many sessile plaques and polyps into the lumen from the underlying tissues.

In most instances the age incidence has been 40 to 60 years of age, with exceptions at 12 and 70 years, the sexes being equally affected.

According to Hiebaum (1934), Luschka described the condition in 1856, a year before the first generally recognized publication by Wilks (1857). The latter found evidence of bony deposits in the larynx, trachea, and bronchi in a 38-year-old man who died of pulmonary tuberculosis.

Dalgaard (1947) reviewed the literature of some 90 cases recorded at that time and included a detailed histological examination of the trachea in a personal case from a man 67 years of age. Since then further reports have been published by Rønnov-Jessen (1952), Pedro (1953), Lell (1953), Padovan (1954), Carr and Olsen (1954), Delord (1954), Moser and Them (1954), Braun and Dhom (1954), Heyden (1954), Dalgaard (1955), Caliceti and Lura (1955), Sors, Rose, Villeneuve, and Cottin (1955), Sobczynski (1955), Simonin, Grilliat, Massinot, and Simon (1957), Israel, Gilbert, and Lechien (1957), Pieri and Casalonga (1957), Hutás and Zsámbéky (1957), and Hempel and Gläser (1958), who collected the largest single number of cases (eight) so far recorded.

Case Report

A man of 61 years of age, a retired civil servant, and in good health previously, developed a left-sided hemiplegia and died the following day in hospital.

Necropsy Report.—The body was that of an elderly man of average build and nutrition, with no external injuries. The surface of the right cerebral hemisphere was flattened, with loss of the normal convolutionary pattern due to an underlying area of infarction in the substance of the right cerebral hemisphere. This was in the distribution of the right middle cerebral artery which was obstructed by atheroma one inch from its origin, the arteries of the brain showing advanced atheromatous changes.

The heart weighed 15 oz. and showed some hypertrophy of the left ventricle. There was no valvular disease, the aorta contained numerous plaques of atheroma and the coronary arteries patchy atheromatous changes without occlusion. The remaining systems of the body, with the exception of the respiratory tract, were quite healthy. The cause of death was cerebral infarction due to cerebral arteriosclerosis.

The upper respiratory tract was healthy and the epiglottis and larynx free from disease. From a point 3 cm. below the level of the vocal cords to the level of the second divisions of the bronchi, the respiratory tract was converted into a narrow triangular-shaped channel, the rigid walls showing numerous bony, hard plaques and polypoidal outgrowths from the underlying tissues (Fig. 1a and 1b). There was no evidence of mucosal ulceration and the membranous portion of the trachea was uninvolved. The normal pattern of the tracheal rings was completely obscured, being identified again only in the bronchial subdivisions. The smaller bronchi were unaffected, and the lungs showed no gross evidence of acute or chronic infection.

Histology.—Sections of the trachea showed the mucosa replaced by a layer of collagen, the submucosal tissues containing evidence of chronic inflammation, including groups of plasma cells and dilated capillary vessels. In the trachea, two distinct layers of partly cartilaginous and partly bony islands were seen. First, immediately beneath the mucosal surface a number of small cartilaginous nodules were evident, some showing calcification and in a few places bone formation (Fig. 2). This layer was intimately related to the internal elastic band of the trachea which showed fragmentation, especially in the neighbourhood of the islands, but at no point did the collagenous fibres enter the newly formed cartilage, nor did the collagenous fibres stain with elastic stains. In the deeper layers, larger islands

FIG. 1a.—Necropsy specimen showing appearance of the trachea and bronchi in tracheopathia osteoplastica.

FIG. 1b.—Radiograph of necropsy specimen showing irregular distribution of calcification in tracheal nodules.

FIG. 2.—Section of tracheal wall with superficial submucosal nodules lying between the tracheal lining and normal cartilage which is evident at one edge. (Van Gieson, × 30.)

FIG. 3a.—Section of the tracheal wall showing the relationship of bony nodules to two normal hyaline tracheal rings. A centrally placed space of adipose tissue is evident in one nodule. (Haematoxylin and eosin, × 12.)

FIG. 3b.—Section of bronchial deposit with well-defined perichondrial membrane. (Haematoxylin and eosin, × 50.)

FIG. 4.—Section of tracheal cartilage merging with the edge of an ectopic bony island, there being no visible perichondrial membrane. (Haematoxylin and eosin, × 125.)

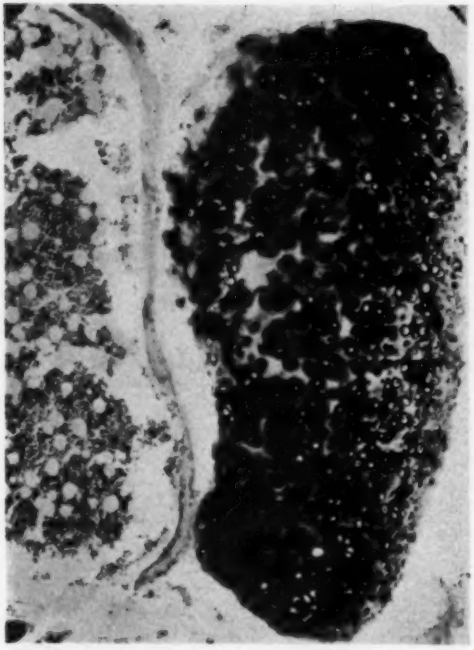

FIG. 5.—Section of bony island showing haemopoietic tissue within the marrow space. (Haematoxylin and eosin, × 120.)

were present (Fig. 3a), many of them osseous and all attached to the superficial aspect of the tracheal rings. In many instances a perichondrial membrane was interposed between the surfaces. This was especially prominent in some of the bronchial deposits (Fig. 3b), but occasionally no such demarcation was evident, the bone merging with the hyaline tracheal cartilage (Fig. 4). In the largest islands a centrally placed space was evident, filled with adipose tissue and normal haemopoietic cells (Fig. 5).

The lungs showed well-marked congestion with fibrinous material in many alveoli as well as evidence of acute inflammation and exudate in the smaller bronchi.

Discussion

The aetiology of the condition is unknown, although numerous theories have been advanced by various writers. These include non-specific chronic infection, tuberculosis, syphilis, mechanical or chemical irritation, and tumour formation. Delord (1954) considered it to be a degenerative process associated with age and chronic infection.

It is likely that infection is coincidental or the result of the condition rather than a factor in its production, and the irritative and tumour theories are not supported by the histological findings.

Two views of more substance are that the condition is the result of an ecchondrosis from the normal tracheal rings, or a metaplasia of the connective tissue of the submucosa of the trachea.

Virchow (1863) considered that the plaques were outgrowths from the tracheal ring, a view supported in later years by serial sections and stereoscopic radiography. However, in many instances the plaques have been crowded into tracheal interspaces, and histological examination has shown no connecting stalk with the tracheal rings, findings that do not support the theory of ecchondrosis.

Aschoff (1910) gave the present name to the condition, regarding it as a metaplasia of the elastic connective tissue cells, whereby undifferentiated connective tissue cells in the superficial layers of the submucosa in close proximity to the elastic fibres became elastic cartilage cells. Other writers, notably Dalgaard (1947), visualized the deposition of calcium in these cells, their invasion by vascular connective tissue to give the so-called "marrow spaces," and their subsequent conversion into bone, following the activity of osteoblasts. No mention is made of haemopoietic tissue in the central spaces, and Pedro (1953) considered the spaces to be submucosal adipose tissue enclosed in the process of formation of an island.

Microscopy in the present case does not support the theory of origin as metaplasia. In the smaller islands elastic connective tissue was found close to the cartilaginous and bony islands, but there was no visible continuity of the elastic threads into the abnormally placed cartilage, and no evidence that the cartilage was elastic in nature. The relationship between the cartilage and elastic tissue appeared to be no more than a geographical one. In the larger, more deeply placed islands, where they were fused with the tracheal rings, and especially where there was no visible perichondrial margin, the appearances suggest an origin from the hyaline tracheal ring.

Clinically the condition is probably not as rare as reports suggest and it is surprising that more instances have not been recorded after bronchoscopy. As far as can be ascertained, there has been no published report in this country since Wilks's original publication. The majority of some 120 cases reported have been found incidentally at necropsy, as in the present instance,

the remainder having been discovered on bronchoscopy, generally in patients with long-standing chronic lung infection and recurrent haemoptysis. The bronchoscopic findings are quite characteristic, the instrument grating and rasping along the surfaces of the bony nodules in the narrowed air passages. Histological examination of material obtained at biopsy has aided diagnosis in some instances.

No specific treatment is available, antibiotics being required where obstruction of the air passages had led to infection. Dalgaard (1955) reported a carcinoma arising in an upper lobe bronchus affected by the condition and regarded this as a complication, an event which could equally well have been fortuitous.

Summary

A case is reported of tracheopathia osteoplastica found incidentally at necropsy. In this rare benign condition of the trachea and bronchi numerous submucosal cartilaginous and osseous nodules project into the lumen causing distortion and narrowing and, where the smaller air passages are involved, chronic lung infection. Histologically ectopic nodules are seen superficially in the submucosal tissues and in the deeper layers, fused to the tracheal rings. The aetiology of the condition remains unknown, although many theories have been proposed including development as an ecchondrosis or as a metaplasia of the elastic connective tissue with subsequent calcification.

I wish to thank Dr. H. G. Broadbridge, the Coroner for the Western Division of Middlesex, for permission to publish, and Professor T. Crawford for advice and the photomicrographs.

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THE INDIRECT HAEMAGGLUTINATION TEST IN DYSENTERY CAUSED BY *SHIGELLA SONNEI* AND *SHIGELLA FLEXNERI*

BY

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The examination of cultures of stools is mostly used in establishing the diagnosis of bacillary dysentery. Agglutination, the most frequently used serological investigation, does not give reliable results and is therefore not generally used.

The bacteriological examination of stools fails in a certain percentage of cases, irrespective of the mode of culture used. Particularly troublesome are examinations of stools in patients treated with antibiotics, for then the stools are usually negative. Patients with negative culture results are admitted to hospital and discharged with a vague diagnosis of gastro-enteritis or entero-colitis. This fact, however, can be very important epidemiologically, especially in children living in collective groups or persons employed in handling food.

Recent work, especially that of Neter and Walker (1954), Neter, Westphal, Lüderitz, and Gorzynski (1956a), and Neter, Gorzynski, Gino, Westphal, and Lüderitz (1956b), demonstrated the value of the haemagglutination technique in the diagnosis of enteric infections, so it was decided to use this method in the investigation of bacillary dysentery.

Material

The sera used in this study came from patients exhibiting symptoms ranging from typical acute dysentery to the asymptomless carrier. *Sh. sonnei* or *Sh. flexneri* was isolated from each individual. In some cases blood was first drawn 12 to 48 hours after the onset of symptoms, while in others two to four weeks had elapsed before the first sample of serum was obtained.

Methods

The techniques originally used were first introduced by Neter and Walker (1954) and Neter *et al.* (1956b), but now the following procedure (Potužník, Kott, and Havlík, 1958) is used.

Polyvalent Antigen *Shigella sonnei*.—Equal portions of agar-grown S and R phase *Sh. sonnei* were suspended in physiological saline. The suspension was centrifuged and the sedimented cells resuspended in saline to a density equivalent to five times that of a 24-hour broth culture. This suspension was heated for about 90 minutes in the steamer and, after cooling, was centrifuged till an opalescent supernatant fluid was obtained which represented the crude antigen ready for use. This was divided into smaller vessels; it was sterilized by boiling in order to prolong its stability when stored at +4° C. The quality of the antigen was always verified by means of an agglutination test with immune rabbit serum against *Shigella sonnei*.

Polyvalent Antigen *Shigella flexneri*.—Equal parts of 24-hour agar cultures of *Shigella flexneri* types 1a, 1b, 2a, 2b, 3, 4a, 4b, 5, and 6 were suspended in physiological saline solution. Final processing was the same as described for the production of the *Shigella sonnei* antigen.

Patients' Sera.—The sera were stored at +4° C. After the diagnosis had been proved bacteriologically the sera were tested with the appropriate antigens.

Erythrocytes.—Human O Rh-negative erythrocytes washed three times and diluted to 10% were mixed with equal amounts of the polyvalent antigen *Shigella sonnei* or *Shigella flexneri* and kept for two hours in a water-bath of 37° C. The modified erythrocytes were then washed three times in physiological saline to remove the excess antigen, which, even in minimal amounts, would produce an inhibitory effect. A 1% suspension of the modified erythrocytes was made which could be stored at +4° C. for as long as a week.

Test Procedure.—To 0.1 ml. amounts of doubling serial dilutions of inactivated serum was added 0.1 ml. of the 1% suspension of modified red cells. After mixing, all the test tubes were centrifuged for two minutes at 800 r.p.m. In a positive case a compact agglutination, which was dispersed only with difficulty, could be separated by gently tapping the test tube.

In a negative case the erythrocytes dispersed easily. Each batch was checked with controls containing both normal and modified erythrocytes in physiological saline solution. Sera containing normal erythrocytes were also examined.

Results

Preliminary experiments were carried out to examine the reliability of the reaction. Thus red blood cells sensitized with either the polyvalent *Sh. sonnei* antigen or the polyvalent *Sh. flexneri* antigen were tested in hyperimmune rabbit serum prepared against various members of the genus *Enterobacteriaceae*. The results are summarized in Tables I–IV and demonstrate that the test is highly specific.

TABLE I

TITRES OBTAINED WITH ERYTHROCYTES SENSITIZED WITH EITHER POLYVALENT *SHIGELLA SONNEI* OR *SHIGELLA FLEXNERI* EXTRACTS AND TESTED IN SALMONELLA RABBIT ANTISERA

Serum Prepared against Salmonella O Group*	Titre Obtained when Tested with Erythrocytes Sensitized with Extract of	
	Sh. sonnei	*Sh. flexneri*
9, 12	1:40±	1:40
4, 5, 12	Negative†	1:80
6, 7	1:20±	1:20
6, 8	Negative	Negative
3, 10	,,	,,
3, 15	,,	,,
Vi	,,	,,

* Serum obtained from Biogena. † Negative = less than 1:20.

TABLE II

TITRES OBTAINED WITH ERYTHROCYTES SENSITIZED WITH EITHER POLYVALENT *SHIGELLA SONNEI* OR *SHIGELLA FLEXNERI* EXTRACTS AND TESTED IN *E. COLI* RABBIT ANTISERA

Serum Prepared against *E. coli* O Group*	Titre Obtained when Tested with Erythrocytes Sensitized with Extract of	
	Sh. sonnei	*Sh. flexneri*
1	Negative†	Negative
2	,,	,,
3	,,	,,
4	,,	,,
5	1:40	1:40
6	Negative	Negative
7	,,	,,
8	,,	,,
9	,,	,,
10	,,	,,
11	,,	,,
12	,,	,,
13	,,	,,
14	1:80	,,
15	Negative	1:40
16	1:80	1:20
17	Negative	Negative
18	1:20	,,
19	Negative	,,
20	,,	,,
21	,,	,,
22	,,	,,
23	,,	,,
24	,,	,,
25	,,	,,

* Serum obtained from Biogena. † Negative = less than 1:20.

TABLE III

TITRES OBTAINED WITH ERYTHROCYTES SENSITIZED WITH EITHER POLYVALENT *SHIGELLA SONNEI* OR *SHIGELL A FLEXNERI* EXTRACTS AND TESTED IN RABBIT *PROTEUS VULGARIS* ANTISERA*

Serum Prepared against *Proteus vulgaris* Group O†	Titre Obtained when Tested with Erythrocytes Sensitized with Extract of	
	Sh. sonnei	*Sh. flexneri*
I, II	Negative‡	Negative
I, III	,,	,,
I, IV	,,	,,
V	,,	,,
VII	,,	,,
VIII	,,	1:80
IX	,,	Negative
X	,,	,,
XII	,,	,,
XIII	,,	,,
XIV	1:20	1:20
XVII, XVIII, XIX	Negative	Negative
XII, XIX, XX	,,	,,
XVII, XVIII	,,	,,
XXI	,,	,,
XXIII	,,	,,
XXIV	,,	,,
XXVIII	,,	,,
XXX	,,	,,
XXXII	,,	,,
XXXIII	,,	,,

* Division according to Kadlecová (Sedlák, 1955). † Serum obtained from Biogena. ‡ Negative = less than 1:20.

TABLE IV

TITRES OBTAINED WITH ERYTHROCYTES SENSITIZED WITH EITHER POLYVALENT *SHIGELLA SONNEI* OR *SHIGELLA FLEXNERI* EXTRACTS AND TESTED IN RABBIT *SHIGELLA* ANTISERA

Serum Prepared against	Titre Obtained when Tested with Erythrocytes Sensitized with Extract of	
	Sh. sonnei	*Sh. flexneri*
Sh. sonnei S+R*	1:2,560	Negative†
Sh. flexneri I (V)†	Negative	1:320
,, ,, II (W)‡	,,	1:160
,, ,, III (Z)‡	,,	1:640
,, ,, IV (103)‡	,,	1:640
,, ,, V (P 119)‡	,,	1:320
,, ,, VI (Newcastle)‡	,,	1:160
,, ,, X‡	,,	1:320
,, ,, Y‡	,,	1:160
Alcalescens‡	,,	1:40
Dispar‡	,,	Negative

* Serum obtained from Biogena. † Negative = less than 1:20. ‡ Serum obtained from Wellcome.

Using a haemagglutination technique 50 samples of blood taken from umbilical veins of newborn infants were tested against *Sh. sonnei* and *Sh. flexneri* antigens. All gave negative reactions.

Neither the time taken for centrifuging nor the speed of centrifugation influenced the result of the reaction.

The sera from 242 individuals from whom either *Sh. sonnei* or *Sh. flexneri* had been isolated were tested. Six hundred and twenty sera from 210 dysenteric patients suffering from Sonne infection were examined by the haemagglutination technique using polyvalent antigen *Sh. sonnei*,

TABLE V
TITRES OF AGGLUTINATING AND HAEMAGGLUTINATING ANTIBODIES IN SERA OF SUBJECTS SUFFERING FROM SONNE OR FLEXNER DYSENTERY

No.	Sera of Subjects with Sonne Dysentery				Sera of Subjects with Flexner Dysentery			
	Titres Obtained with *Sh. sonnei* Antigen		Titres Obtained with *Sh. flexneri* Antigen		Titres Obtained with *Sh. flexneri* Antigen		Titres Obtained with *Sh. sonnei* Antigen	
	Agglutination	Haemagglutination	Agglutination	Haemagglutination	Agglutination	Haemagglutination	Agglutination	Haemagglutination
1	Negative*	1:50	Negative	Negative	Negative	1:50	Negative	Negative
2	,,	1:100	,,	,,	,,	1:50	,,	1:50
3	,,	1:100	1:20	,,	,,	1:100	,,	1:50
4	,,	1:150	Negative	,,	,,	1:150	,,	1:50
5	,,	1:200	,,	1:50	,,	1:200	,,	Negative
6	1:20	1:600	,,	Negative	,,	1:300	1:20	1:100
7	1:40	1:400	,,	,,	1:20	1:300	1:20	1:100
8	1:40	1:600	,,	,,	1:40	1:300	1:20	1:100
9	1:80	1:300	,,	,,	1:80	1:200	1:20	1:50
10	1:80	1:600	,,	,,	1:80	1:400	Negative	Negative
11	1:160	1:300	,,	,,	1:80	1:600	1:20	1:50
12	1:160	1:1,200	,,	,,	1:320	1:2,400	Negative	1:150

* Negative—by agglutination less than 1:20; by haemagglutination less than 1:50.

and 69 sera from 32 dysenteric patients with Flexner infection were examined by the same technique using polyvalent antigen *Sh. flexneri*. Finally 12 sera from individuals suffering from Sonne shigellosis and 12 sera from individuals suffering from Flexner shigellosis were examined using haemagglutination and the Widal technique with both polyvalent antigens in both groups of sera (Table V). The agglutinin titres, using the Widal technique, were either negative or low, and always lower than the titres obtained by the haemagglutination test. Some sera gave positive titres against both shigellas; we believe that these reactions are specific and are due to previous dysentery infection with the other type of Shigella.

In the group of 12 patients all suffering from Flexner dysentery (Table V) positive haem-agglutinin titres against *Sh. sonnei* were often found. In repeated tests *Sh. flexneri* haemagglutinins were markedly unstable, whereas the *Sh. sonnei* haemagglutinins did not change. These patients were all children from a group living in an institution where Sonne dysentery had been found some time before. During the period in hospital an average of three samples of serum at five-day intervals was taken from these patients.

The titre 1:50 is thought suggestive of infection, whereas the titre 1:100 and higher is accepted as positive. Obviously only repeated examinations are of diagnostic value, the rise in antibody being very significant. Suggestive titres usually appeared about six to eight days after the onset of symptoms and remained positive for several weeks at least. It may be stressed that even in mild attacks of the disease and in so-called asymptomatic carriers positive antibody levels could be demonstrated in the majority of cases.

As can be seen in Table VI most sera showed a positive or at least a suggestive result in the first specimen; in the later specimens the titres were

TABLE VI
TITRES OF HAEMAGGLUTINATING ANTIBODIES IN SERA OF SUBJECTS SUFFERING FROM SONNE OR FLEXNER DYSENTERY

Titre	Sera of Subjects with Sonne Dysentery					Sera of Subjects with Flexner Dysentery				
	Intervals of Blood Samples from Beginning of Illness				No. of Examined Sera	Intervals of Blood Samples from Beginning of Illness				No. of Sera Examined
	1–7 Days	8–14 Days	15–42 Days	More		1–7 Days	8–14 Days	15–42 Days	More	
Negative*	26	5	10	12	53	6	1	0	0	7
1:50	15	29	36	11	91	4	3	2	0	99
1:100	9	46	81	13	149	3	5	5	2	15
1:200	4	31	95	13	143	1	3	6	2	12
1:300	5	28	35	2	70	1	2	5	1	
1:400	7	16	28	3	54	0	2	4	0	6
1:600	6	19	10	2	37	1	1	2	1	5
1:800	1	7	4	0	12	0	1	0	0	1
Higher	0	7	3	1	11	1	2	2	0	5
Total	73	188	302	57	620	17	20	26	6	69

* Negative—less than 1:50.

positive. Of the sera with high titres, 1:600 and higher, no direct relationship was shown to exist between the high titres and either the severity of the illness or the treatment. The titres obtained are shown in Table VI.

Discussion

In the past bacillary dysentery has not been diagnosed by the demonstration of agglutination in patients' sera, first because positive results are obtained in diseases other than bacillary dysentery, and secondly because a large number of patients fail to develop detectable agglutinins. Cruickshank and Swyer (1940) showed that a rise in titre occurred in patients with Sonne dysentery, and Seeliger (1953) suggests a certain diagnostic significance to agglutination tests in patients whose disease is caused by *Sh. shigae, Sh. schmitzi, Sh. boydii,* and *Sh. sonnei,* non-specific reactions being exceptional in these infections.

On the other hand, Seeliger considers the evaluation of positive titres against *Sh. flexneri* as difficult, since this group is closely related antigenically to a number of Gram-negative rods.

The haemagglutination test in dysentery was first used by Neter and Walker (1954), who showed that specific titres were obtained even in the acute phase of the disease and that the titre remained high for a considerable length of time (Neter and Dunphy, 1957).

Another advantage of the haemagglutination test is that erythrocytes are able to adsorb fully on their surface several antigens simultaneously (Hayes, 1951; Neter, Bertram, and Arbesman, 1952; Landy, 1954).

Neter, Westphal, Lüderitz, and Gorzynski (1956a), Neter *et al.* (1956b), and Neter and Dunphy (1957) used polyvalent sensitized erythrocytes for the diagnosis of various diseases due to *Enterobacteriaceae,* to which the dysenteries also belong.

The results presented in this study show that the haemagglutination procedure is highly specific and more sensitive than agglutination and should therefore be used in the diagnosis of bacillary dysentery.

It is not suggested that this serological technique should be used to the exclusion of cultural techniques; nevertheless haemagglutination can be an important diagnostic tool, especially in patients in whom therapy has started and stool cultures are negative.

Summary

From control tests and the results of examining 689 sera from patients suffering from Sonne or Flexner dysentery proved by culture, the following conclusions may be drawn:

(1) The haemagglutination test demonstrates raised specific titres in the sera of dysenteric patients where *Sh. sonnei* or *Sh. flexneri* was the infecting agent.

(2) A titre of 1:50 is regarded as suspicious, 1:100 and higher as positive.

(3) Repeated examinations of patients' sera show a rise in Shigella agglutinins during the course of the disease.

(4) Haemagglutination antibodies are present in the sera of patients with atypical disease and in symptomless carriers.

(5) The haemagglutination technique is particularly valuable as a means of proof of the disease in patients undergoing treatment with antibiotics and whose faeces yield negative cultures.

(6) The haemagglutination test does not replace the need for isolation of the causal organism.

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A SIMPLE FILTER-PAPER DISC METHOD FOR DETERMINING THE SENSITIVITY OF *MYCO. TUBERCULOSIS*

BY

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Because of the success of modern chemotherapy in tuberculosis, it has become necessary to estimate the drug sensitivity of most strains of *Myco. tuberculosis* isolated from patients. What has been until recently a specialist investigation is now a routine test performed in a general hospital laboratory.

With this transition from a research to a routine laboratory procedure, certain modifications of existing methods are worth considering, not only to obtain economy in time, media, glassware, and incubator space, but also to reduce the risks of infection of laboratory staff.

Drug sensitivity of *Myco. tuberculosis* can be determined in fluid media (Medical Research Council, 1953) or by slide culture technique (Rubbo and Morris, 1951), but both methods are time consuming and hazardous to the operator, and the former uses much incubator space. More commonly, solid media impregnated with various concentrations of appropriate drugs are used, the aim being to determine the minimum drug concentration which inhibits growth of a test strain and of a sensitive control. This method has the disadvantages that the gaps between drug concentrations are wide, making interpretation of results sometimes difficult, and that several slopes of media are required for each drug investigated.

Hoyt (1951) reported the use of compressed tablets of streptomycin in performing sensitivity tests. Collins (1955, 1956) used filter paper discs impregnated with streptomycin or isoniazid, placed upon a solid medium, in parallel with tube dilution in Dubos liquid medium, and found close correlation between them. In the present investigation, we have performed sensitivity tests on organisms using impregnated paper discs, and have compared the results of this method with those obtained using impregnated solid media in parallel.

Materials

First, 94 cultures of *Myco. tuberculosis*, isolated principally from sputum, were tested for sensitivity to streptomycin, then 205 cultures were tested for sensitivity to streptomycin, P.A.S., and isoniazid. Later 49 and 52 cultures were tested with "dipasic" and "viomycin," respectively.

Fifty-seven positive sputum concentrates were tested directly with streptomycin, P.A.S., and isoniazid.

Methods

Culture Media.—All primary cultures were grown on Löwenstein–Jensen medium (Mackie and McCartney, 1953) dispensed in 7 ml. amounts in 1 oz. (30 ml.) screw-capped bottles sloped at 10° to the horizontal during inspissation. The same medium was used in performing tests with paper discs.

The impregnated Löwenstein–Jensen slopes used were obtained from the Southern Group Laboratory in ½ oz. vials.

Inoculum.—Several colonies from each primary culture were picked off with a "spade" of flattened nichrome wire, and emulsified on the dry inside of a sterile bijou bottle above 1 ml. of distilled water. The emulsion was mixed to a smooth suspension, and, by dilution with water, matched with No. 1 Brown's opacity tube (500×10^6 organisms per ml.). One drop of this suspension, approximately 0.015 ml., was delivered from a sterile pipette terminating in a No. 19 gauge hypodermic needle, and allowed to run down the centre of each culture slope, which was then capped and incubated upright without spreading the inoculum.

Concentrates.—Sputa, homogenized by 3% NaOH for 20 minutes and neutralized by 10% HCl, were centrifuged at 3,000 r.p.m. for 30 minutes. The inoculum was 0.015 ml. of the neutral deposit. Only concentrates containing at least one bacillus per microscopic field of the 1/7 in. (3.6 mm.) oil-immersion lens (500×) were accepted for the direct test.

TABLE I
COMPARISON OF IMPREGNATED MEDIA AND DISC METHOD TESTS WITH VARIOUS STRAINS

Drug	Impregnated Media Maximum Concentration per ml. Permitting Growth				Disc Result	Zones of Inhibition (mm.)			Total Strains Tested
						Range	Range of Majority	Mean	
Streptomycin ..	< 1 µg.	1 µg.	3 µg.	10 µg.					
	229	16	8 13	33	S PR R	10–24 5–7	12–18 (84%)	14·5 6·5	299
P.A.S.	< 2 µg.	2 µg.	10 µg.						
	177	7 10	11		S PR R	10–50* 6–9	20–40 (75%)	29 7	205
Isoniazid	< 0·2 µg.	0·2 µg.	1 µg.	5 µg.					
	148	6 3	14	34	S PR R	10–50† 6–8	25–40 (85%)	33 7	205
Dipasic	< 0·2 µg.	0·2 µg.	1 µg.	5 µg.					
	32	2	4	11	S R	10–50		28	49
Viomycin	< 10 µg.	10 µg.	50 µg.						
	45	4	3		S PR R	10–24 6–9	10–16 (91%)	12 7	52

S = Sensitive. PR = Partially resistant. R = Resistant. * Twenty-four strains were completely inhibited. † Six strains were completely inhibited.

Discs.—Discs, each of 6 mm. diameter, were cut from Ford 428 mill coloured blotting-paper, sterilized by dry heat, impregnated with the appropriate drug solution, and dried *in vacuo* in a desiccator over phosphorus pentoxide. The prepared discs showed no loss of potency when stored at 4° C. for 12 months. A simple colour code distinguished the drugs.

The amount of drug used in each type of disc was: streptomycin 10 µg., P.A.S. 5 µg., isoniazid 1 µg., "dipasic" 1 µg., and viomycin 25 µg. The drug concentrations per disc were selected by experiment in order that (*a*) a zone of at least 10 mm. occurred with strains accepted as sensitive by impregnated media ; (*b*) no zone was obtained with strains accepted as resistant by impregnated media ; and (*c*) a zone of less than 10 mm. occurred with partially resistant strains.

In testing, a disc was placed on the Löwenstein slope, near the top of the medium, as soon as convenient after seeding the culture suspension.

Zones were obtained by measurement of the radius from the centre of the disc to the edge of the inhibition of growth. Measurements were made from both sides of the slope and their average accepted.

Period of Incubation.—With discs it was possible to record sensitivities in 10 to 14 days, and no encroachment on the zone of inhibition occurred within 21 days. Growth on impregnated media was recorded at 21 days, and an organism was considered sensitive when less than 20 colonies grew on a slope.

Results

Comparison of Discs with Impregnated Media.—From Table I it can be seen that there were no discrepancies between the methods performed in parallel. With all the drugs used, the 10 mm. zone of inhibition was found to provide a yardstick for sensitive strains.

Reliability of Discs.—Tables II and III show that results using discs are reliable with fully sensitive strains (Table II) and with strains of diverse sensitivity (Table III). The three tests made on each strain, illustrated in Table III, were carried out on slopes from the same batch of

TABLE II
IMPREGNATED MEDIA AND DISC METHOD TESTS WITH SENSITIVE H37Rv STRAIN

Drug	Total No. of Tests	Impregnated Media Maximum Concentration Permitting Growth (µg./ml.)	Zones of Inhibition (mm.)	
			Range	Mean
Streptomycin	28	< 1	13–16	14·5
P.A.S. ..	28	< 2	15–45*	28
Isoniazid ..	28	< 0·2	25–45†	31
Dipasic ..	24	< 0·2	15–45	30
Viomycin ..	24	< 10	10–16	12

* In four tests growth was completely inhibited. † In one test growth was completely inhibited.

medium, and were seeded on the same day with equal inocula from the same suspension of each strain. Under these conditions zone variation between tests was almost eliminated.

Effect of Size of Inoculum.—Highly concentrated inocula produced results indicating false resistance, and a too dilute inoculum gave results of false sensitivity. Typical results are shown in Table IV. For these tests, the inoculum, which was always 0.015 ml., was altered from the standard (No. 1 Brown's opacity tube) to give 0.1×, 10×, and 100× the concentration of bacteria.

Discussion

The two methods, impregnated media and impregnated discs, show close correlation and no advantageous features in the use of impregnated media. In fact, because of the wide spacing of concentrations in impregnated media, discs appeared to give clearer results with strains showing partial resistance. For example, nine strains resisting 0.2 μg./ml. of isoniazid but sensitive to 1.0 μg./ml. were separated by the disc technique into six strains partially resistant and three resistant.

Mixed strains of differing sensitivities in a culture can be detected more readily by discs than by impregnated media. In one test isoniazid inhibited most of the growth at 25 mm., but 50 colonies grew within the zone: the latter on subculture proved to be uniformly resistant. The readings with impregnated media suggested doubtful sensitivity. Inocula from only one colony of the primary culture are therefore quite unreliable for sensitivity testing.

The mean zones of the H37Rv strain (Table II) and of other sensitive strains (Table I) compared closely, although zones of much greater range occurred with the latter, confirming that the degree of sensitivity does influence zone size. The repeated testing of H37Rv, however, showed variations which suggest that it would be unwise to construct standard graphs to convert zones into μg./ml. as suggested by Maccabe and Gould (1954). It seems likely that zones were also influenced by batch-to-batch differences in media. Zone variations between tests were insignificant when tests were carried out under as nearly identical conditions as possible (Table III).

Maccabe and Gould (1954) deduced that zones were altered by not more than 1 mm. by variations some tenfold in the inoculum, and Middlebrook (1954) that a concentrated inoculum may cause a fully sensitive strain to simulate complete resistance. These observations are borne out in Table IV where it is shown that the inoculum should be no more dilute than the "standard" nor concentrated more than tenfold. This standard was found to be both reliable and reproducible.

Sensitivity Testing of Concentrates.—This was of little value and the best results were obtained from pre-treatment specimens. During a course of chemotherapy, heavily positive concentrates yielded insufficient growth for the interpretation

TABLE III

DISC METHOD TESTS IN TRIPLICATE UNDER IDENTICAL CONDITIONS

Strain	Streptomycin				P.A.S.				Isoniazid			
	Result	Zone (mm.)			Result	Zone (mm.)			Result	Zone (mm.)		
		1	2	3		1	2	3		1	2	3
998	PR	7	8	8	R	–	–	–	S	19	16	20
1034	S	12	12	13	S	20	20	18	S	30	38	30
1062	R	–	–	–	S	22	22	20	R	–	–	–
1151	R	–	–	–	R	–	–	–	R	–	–	–
1182	S	12	12	13	S	36	+	+	S	38	35	41
1313	PR	6	8	7	S	15	15	17	S	27	30	33

+ Growth was completely inhibited. – Denotes absence of inhibition.

TABLE IV

DISC METHOD TESTS WITH VARYING INOCULA (ZONES IN MM.)

Strain	Streptomycin				P.A.S.				Isoniazid			
	0·1×	Standard*	10×	100×	0·1×	Standard	10×	100×	0·1×	Standard	10×	100×
1151	8	–	–	–	†	–	–	–	–	–	–	–
1182	13	12	13	–	†	36	35	–		38	30	–

* Standard equivalent to No. 1 Brown opacity tube. † Growth was completely inhibited. – Denotes absence of inhibition.

of sensitivity. Uneven distribution of viable bacilli is, however, easily detected by the disc technique since some growth is expected on each slope, whereas a chance distribution of negative slopes of impregnated media may lead to a false sensitivity report.

The disc method is readily adapted for testing strains of *Myco. tuberculosis* against other drugs, and a number of tests have been performed with terramycin (25 μg./disc) and cycloserine (50 μg./disc).

Since this material was collected, more than 700 strains of *Myco. tuberculosis* have been tested for sensitivity by the disc method, without any apparent difficulties. Promising results for combinations of drugs have been obtained by placing discs containing different drugs on top of each other, and this is being further investigated.

Summary

A simple and reliable method of testing the sensitivity of *Myco. tuberculosis* to various drugs is described. Discs containing a single strength of each drug are placed at the upper ends of Löwenstein–Jensen slopes seeded with standard inocula of strains under test.

The method has many advantages over the use of impregnated media, not the least being a substantial reduction in the number of slopes. For doubtfully resistant strains interpretation has been more exact.

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A COMPARISON OF THE SHEEP CELL AND LATEX AGGLUTINATION TESTS IN RHEUMATOID ARTHRITIS

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The serum of patients with rheumatoid arthritis contains a factor which causes agglutination of sensitized particulate bodies. This phenomenon has been demonstrated using sheep red cells (Waaler, 1940), collodion particles (Wallis, 1946), bacteria (Wager, 1950), polysterene latex particles (Singer and Plotz, 1956), and bentonite (Bozicevich, Bunim, Freund, and Ward, 1958). Rose, Ragan, Pearce, and Lipman (1948) devised a diagnostic test using sensitized sheep red cells, since when a considerable literature has appeared describing modifications of this test, its clinical significance, and the mechanisms involved (Ziff, 1957; Kellgren and Ball, 1959).

Because of its relative simplicity the latex test has recently attracted more attention and a number of modifications have been described, including the use of a glycine buffer in the place of the original borate buffer (Egghart, Wiedermann, and Braunsteiner, 1957; Valkenburg and Mos, 1958), a drop dilution technique (Rothermich and Philips, 1957), and the use of the patient's own gamma globulin (Singer and Plotz, 1958a). Bartfeld, Mahood, and Hartung (1958) and Burby and Behr (1958) have compared the results of sheep cell agglutination tests and the latex test. A slide agglutination test has recently been described (Singer and Plotz, 1958b).

The object of the present investigation was to compare the results of sheep cell agglutination tests and latex tests performed in parallel on sera from a large physical medicine department, with the possibility of adopting the much quicker and simpler latex test in place of the sheep cell test.

Method

Ball's (1950) modification of the sheep cell test was used. The test consists essentially of inactivating the complement in the test serum, absorbing the naturally occurring sheep cell agglutinins, and finally adding sheep cells sensitized with rabbit anti-sheep serum to serial dilutions of the decomplemented absorbed serum. After incubation at 37° C. for one hour and refrigeration at 4° C. overnight, the tubes are read for agglutination of the sensitized sheep cells by the "rheumatoid factor." A titre of 1 in 16 or over was interpreted as a positive test.

The latex agglutination tests were performed essentially as originally described by Singer and Plotz (1956). Polyvinyl toluene latex particles (Dow Chemical Company, Michigan, U.S.A.) of particle size 0.81 μ were mixed with human gamma globulin and added to serial dilutions of the test serum from 1 in 20 to 1 in 10,240. After incubation for two hours at 56° C. the tubes were read for agglutination of the particles and a titre of 1 in 20 or over interpreted as a positive test. It was found that after two hours' incubation the tests could be read easily with the aid of a small-aperture transmitted light. Centrifugation and refrigeration before reading, as described by Singer and Plotz (1956), was unnecessary. In a positive test a prozone was frequently noted, with only slight agglutination in the first tube, increasing through the second tube to reach maximal agglutination in the third tube, the agglutination then progressively diminishing to the end-point.

Results

Five hundred and four sheep cell and latex agglutination tests were performed in parallel. The results of the test have been arranged in four groups as follows:

Group I	... ...	Both tests positive in 157 cases
Group II	... ...	Rose test positive and latex test negative in 20 cases
Group III	... ...	Rose test negative and latex test positive in 16 cases
Group IV	... ...	Both tests negative in 311 cases

The two tests corresponded in 468 or 93% of tests.

All the patients with rheumatoid arthritis were again classified according to the American Rheumatism Association criteria (Ropes, Bennett, Cobb, Jacox, and Jessar, 1956).

The 157 cases in Group I were classified as rheumatoid arthritis in 151, probable rheumatoid arthritis in one, possible rheumatoid arthritis in two, rheumatoid arthritis with psoriasis in two, and collagen disease in one. The patient with probable rheumatoid arthritis subsequently developed sufficient criteria to make the diagnosis "definite." The two cases of rheumatoid arthritis with psoriasis were typical rheumatoid with psoriasis and not cases of psoriatic arthritis; in the final analysis of the rheumatoid material these two cases are therefore included. In spite of a necropsy a final diagnosis was not reached in the case labelled "collagen disease."

Comparison of the titres of the Rose tests and latex tests suggests a tendency for the mid to high Rose titres to be associated with high latex titres. It was not possible, however, to predict the titre of one by knowing the titre of the other. When positive, latex titres were never below 80 and usually higher (Table I).

TABLE I
COMPARISON OF 157 POSITIVE ROSE AND LATEX TESTS BY TITRE

Rose Tests Total	4	9	13	19	25	30	21	36	157
2,048	—	—	—	—	1	1	1	3	6
1,024	—	—	—	—	—	—	—	3	3
512	—	—	—	—	2	3	2	4	11
256	—	—	1	1	3	5	2	9	21
128	—	3	3	4	3	4	6	9	32
64	—	2	3	4	5	9	2	4	29
32	—	3	3	5	8	3	7	3	32
16	4	1	3	5	3	5	1	1	23
Latex tests	80	160	320	640	1,280	2,560	5,120	10,240	157

Group II, with a positive Rose test and negative latex test, included 15 cases of rheumatoid arthritis, one of probable rheumatoid arthritis, one of psoriatic arthropathy, one of sacro-iliitis, and one definite and one probable case of disseminated lupus erythematosus. Nine of the patients with rheumatoid arthritis were "classical" cases and in the other six it was just possible to make a diagnosis of definite rheumatoid arthritis; in two of these latter cases the Rose test was the fifth criterion, although further criteria subsequently developed. The patient with psoriatic arthropathy was typical, with terminal involvement of interphalangeal joints. Both patients with disseminated lupus were early cases without locomotor signs. The patient with sacro-iliitis has had a persistently positive Rose test, no radiological evidence of spondylitis, and radiotherapy has been ineffective. The titres of the Rose test in this group were mostly low, with nine at 1 in 16, eight at 1 in 32, one at 1 in 64, and two at 1 in 128. Both the patients with a titre of 1 in 128 had classical rheumatoid arthritis.

Group III, with a positive latex test and negative Rose test, included 10 cases of rheumatoid arthritis, one of probable rheumatoid arthritis, one of possible rheumatoid arthritis, one of arthritis with psoriasis, one of Reiter's disease, one of the shoulder–hand syndrome, and one case of uveitis. Eight of the 10 definite rheumatoid cases were classical, one had a history of six months and five criteria without the latex test, and one a short history of joint involvement and a previous history of suspected but unconfirmed discoid lupus. The case classified as probable rheumatoid arthritis presented with rupture of the extensor tendons of the hand due to attrition. The patient with possible rheumatoid arthritis gave a very short history, and all investigations other than the latex test were negative. The case of arthritis and psoriasis had a long history of psoriasis and a brief history of joint pain and swelling; he subsequently died with acute nephritis.

Group IV, with both tests negative, contained over 25 conditions but included 81 patients with a diagnosis of rheumatoid arthritis.

In all, sera from 259 rheumatoid patients were tested, both tests being positive in 153, the Rose test positive and the latex negative in 15, the Rose test negative and the latex positive in 10, and both tests negative in 81. There was therefore agreement in 234 rheumatoid sera out of a total of 259, or in 90%. Considering Groups II and III, the overall results were:

Both tests positive	153 (59%)
Rose test positive	168 (64.8%)
Latex test positive	163 (62.9%)
Either test positive	178 (68.7%)

Discussion

It is clear that the results of the sheep-cell and latex agglutination tests coincide in the majority of cases, with each test having approximately the same sensitivity. If both tests were performed together this would result in a slightly higher total of positive serology, but the slight advantage gained would be out of proportion to the work involved. The latex test is much quicker and simpler to perform, 20 or more tests being easily set up and read in a morning. One possible disadvantage of replacing the sheep cell test by the latex test may be that the titre of the sheep cell test has been related to the prognosis of the disease (Duthie, Brown, Knox, and Thompson, 1957): there seems to be no correlation between

the titre of the sheep cell tests and latex tests in this series (Table I).

It would be useful to have a rapid screening test for the "rheumatoid factor" in serum. In devising such a test, either on a slide or in tubes, the prozone phenomenon noted in the latex test must be borne in mind.

Summary

Five hundred and four sheep cell and latex agglutination tests were performed in parallel and the two tests gave coincidental positive or negative results in 93%. There was no correlation between the titres of positive tests. It is suggested that the latex test is a satisfactory simple alternative to the sheep cell test in routine diagnostic work.

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THE SIGNIFICANCE OF HOWELL-JOLLY BODIES IN RED CELL PRECURSORS

BY

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During the routine examination of sternal marrow smears it was observed that the presence of nuclear fragments in red cell precursors, commonly known as Howell–Jolly bodies, was usually associated with clear evidence of vitamin B_{12} or folic acid deficiency. Moreover, this seemed to be so even when the megaloblastic transformation of erythropoiesis was not well developed. With further experience the presence of Howell–Jolly bodies does appear to be a finding of diagnostic value even in doubtful cases. In order to test the validity of this impression a series of sternal marrow films examined for Howell–Jolly bodies has been reviewed. In this the incidence in iron deficiency and other states where B_{12} or folic acid deficiency could clearly be excluded has been compared with the incidence in conditions known to be associated with B_{12} or folic acid deficiency. The results of this investigation are presented here.

Recognition of Howell–Jolly Bodies

In Leishman-stained marrow smears the typical morphology is shown in the illustration. When multiple bodies of substantial size were present their appearance was unmistakable and there was no difficulty in interpretation. When, however, the structures were single and very small it was difficult to know whether they might not be an artefact of some sort or perhaps Pappenheimer bodies (see later). Since the structures were never numerous it was not possible to employ the Feulgen and Prussian blue reactions as aids to positive identification in such cases.

Iron-deficiency Anaemia

Fifty cases were collected from which sternal marrow had been obtained and the anaemia had been conclusively shown to be the result of iron deficiency by peripheral blood findings, absence of stainable iron in the marrow sections (Hutchison, 1953), and response to iron therapy alone. In only one case could infrequent erythroblasts with Howell–Jolly bodies be found. This patient was in a poor nutritional state and had ulcerative colitis. In three other smears occasional tiny granules of doubtful nature were found, apparently in erythroblasts.

Pernicious Anaemia

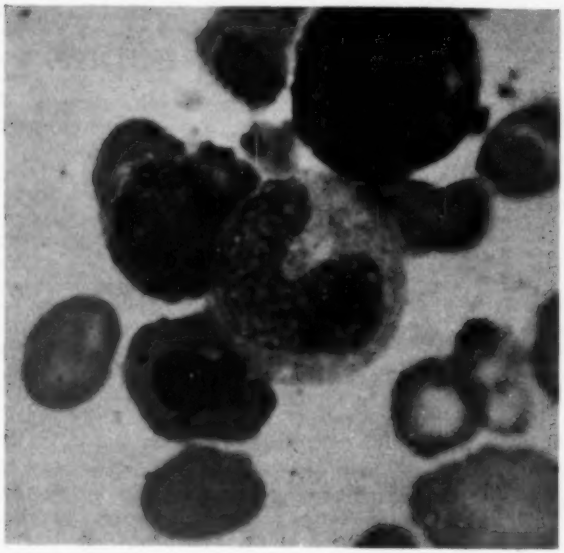
Howell–Jolly bodies were readily identified in a varying proportion of megaloblasts in each of 52 consecutive cases of untreated classical Addisonian anaemia. They could be found during the preliminary scanning of the marrow smear within a period of two or three minutes in all but a very few instances. The number of megaloblasts containing these bodies seemed to be directly proportional to the degree of megaloblastic transformation and the severity of the anaemia.

Other Macrocytic Anaemias

As in pernicious anaemia, Howell–Jolly bodies were found constantly in association with megaloblastic erythropoiesis. Thus positive findings were obtained in seven patients with the malabsorption syndrome, two post-gastrectomy macrocytic anaemias, four cases of megaloblastic anaemia of pregnancy, six examples of incompletely investigated macrocytic anaemia in which the fundamental abnormality was not demonstrated, and in two cases of macrocytic anaemia after treatment with the anticonvulsant drug mysoline. Howell–Jolly bodies were also observed in two cases of severe haemolytic anaemia, and in a case of portal hypertension; once again erythropoiesis showed other evidence of megaloblastic transformation in all three cases.

Miscellaneous Anaemias

Forty marrows were reviewed for Howell–Jolly bodies. These were all cases in which stainable iron was present and there was nothing in the report on the marrow cytology to indicate that megaloblastic transformation had been suspected.



Many of these were not from primary disorders of the haemopoietic system and covered a wide variety of diseases. In 32 instances no Howell–Jolly bodies could be found; four were classified as doubtful where solitary small granules, possibly artefacts, were seen, and four were found to show infrequent granules morphologically indistinguishable from Howell–Jolly bodies. The case histories and course of these eight patients were reviewed. No clear lead was obtained in the first group of four doubtful positives, whereas in the second group of four the records proved most interesting. One was finally considered to have had a spontaneous partial remission of Addisonian anaemia; another was found to have had megaloblastic anaemia during pregnancy months previously; the third was being treated for a resistant urinary tract infection when a "normocytic" anaemia was discovered. The haemoglobin rose from 60 to 75% in 14 days on folic acid. The last patient is still under investigation.

Relationship to Splenectomy

After splenectomy in four cases of haemolytic anaemia and in two cases of splenic anaemia (Banti's syndrome) single bodies similar to Howell–Jolly bodies were seen in the erythrocytes, but no typical nuclear remnants could be identified in the red cell precursors in two cases in which marrow was available. This is in sharp contrast with their distribution in B_{12} or folic acid deficiency states. Many of the erythrocytes containing these single bodies were siderocytes (Pappenheimer bodies) and this was confirmed by positive iron and negative Feulgen reactions in two of the cases.

Comment

The presence of Howell–Jolly bodies in the erythroblasts has been found to be a useful aid in the routine interpretation of sternal marrow smears. Where megaloblastic transformation is not well developed the finding of Howell–Jolly bodies provides helpful confirmatory evidence of the tendency and suggests the need for further investigation along those lines, e.g., in Addisonian anaemia presenting with neurological changes or glossitis and minimal haematological involvement. In such cases their presence encourages further investigation by B_{12} assay, the Schilling test, or folic acid clearance.

It has not been our purpose to describe the appearance of Howell–Jolly bodies in the peripheral blood, although most of the reported work on these structures deals largely with this aspect. Thus most textbooks contain references to the invariable finding of Howell–Jolly bodies in a proportion of the erythrocytes after splenectomy (Pepper and Austin, 1943; Dacie, 1954; Wintrobe, 1956; Whitby and Britton, 1957), in cases of splenic atrophy (Shur, 1908, and Schilling, 1924, see Boveri; Hirschfeld and Dünner, 1933; Boveri, 1942; Leitner, 1949), and in congenital absence of the spleen (Bush and Ainger, 1955; Wintrobe, 1956). These bodies are also said to occur frequently in severe secondary anaemia (Pepper and Austin, 1943; Whitby and Britton, 1957), in haemolytic anaemia (Pepper and Austin, 1943; Whitby and Britton, 1957), in thalassaemia (Whitby and Britton, 1957), and in leukaemia (Pepper and Austin, 1943; Whitby and Britton, 1957), as well as in pernicious anaemia (Morris, 1907) and sprue (Boveri, 1942), but no studies of the conditions of their occurrence in red-cell precursors appears to have been made. It is interesting to note the frequency of an associated megaloblastic anaemia in the case notes recorded by some investigators (see, for example, Boveri, 1942), and the confusion which often exists in the differentiation of Pappenheimer and Howell–Jolly bodies (Pepper and Austin, 1943). It is even doubtful if the structures described originally by Howell in 1890 are

identical with what are now commonly accepted as Howell–Jolly bodies.

With regard to the origin of these bodies, it is likely that they are produced by abnormal mitosis in the late megaloblasts when single chromosomes or groups of chromosomes become detached, fail to take part in the formation of the interphase nucleus, and remain free in the cytoplasm as nuclear remnants.

It seems probable that disordered nucleic acid synthesis resulting from B_{12} or folic acid deficiency is the fundamental factor in the production of this abnormal behaviour at mitosis.

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A HISTOCHEMICAL INVESTIGATION OF THE LEUCOCYTES IN KWASHIORKOR*

BY

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The present investigation was carried out in conjunction with a series of biochemical and haematological studies of the blood in kwashiorkor conducted at the Pretoria General Hospital (Kinnear and Pretorius, 1956 and 1957) in order to extend the overall picture of the state of various tissues in this malnutrition syndrome. For a complete review of the application of histochemical procedures to leucocytes the reader is referred to Wachstein (1955).

Material and Methods

In the present investigation histochemical tests for lipids, ribonucleic acid (RNA), desoxyribonucleic acid (DNA), glycogen, mucoproteins, peroxidase, and alkaline phosphatase were carried out.

The tests were performed on smears prepared from samples of peripheral blood taken from each of 20 Bantu infants suffering from kwashiorkor. The tests were carried out at the beginning of treatment and at weekly intervals thereafter. The average number of smears studied per patient was five, but in some cases smears were obtained over a period of 11 weeks. In all, about 500 separate histochemical tests were made.

Blood smears taken from 10 healthy Bantu children were used as controls and, to afford a further comparison, smears from eight adult whites were also examined.

The "scoring" method of Kaplow (1955) was adopted in order to enable the intracellular alkaline phosphatase activities of blood smears from different individuals to be evaluated and compared. This method involves the grading of 100 successive leucocytes according to colour intensity to provide an index of phosphatase activity. The range of scores employed is from 100 to 400. Kaplow was able to demonstrate alkaline phosphatase activity only in neutrophils, and scoring for alkaline phosphatase activity was therefore confined to neutrophils.

The same scoring method was also applied to evaluate lipid content and peroxidase activity, granulocytes being tested in these cases and the degree of granulation being recorded instead of colour intensity. Indications of fat or peroxidase in monocytes were disregarded. In the case of glycogen, RNA, DNA, and mucoproteins, only qualitative judgments were made of all types of leucocytes.

The shortcomings of subjective quantitation with histochemical methods are obvious, and the application of such methods is open to criticism. In order to reduce the errors as much as possible, strict attention was paid to standardization. The time of treatment of the preparations, the age of the reagents, and the temperature at which they were kept were maintained as uniform as possible. The stain for lipids (Sudan black B) was not used if older than three days. All observations from the slides were made by one author only.

It was considered advisable to confine scoring to a portion of each slide where the thickness of the smear was even; this entailed disregarding the "tail" of the smear. Readings were taken along rows on the slide, according to the "strip" method.

The following techniques were used:

Lipids.—Sudan black B and May-Grünwald-Giemsa technique (Baillif and Kimbrough, 1947).

Peroxidase.—Copper sulphate method of Sato and Sekiya, as described by Bessis (1956), using safranine as counterstain.

DNA.—Feulgen reaction, as described by Darmady and Davenport (1954).

RNA.—Unna-Pappenheim stain, as described by White (1947). Control smears were treated with ribonuclease in veronal acetate buffer at *p*H 6.8.

Glycogen. — Periodic-acid-Schiff method, as described by Hotchkiss (1948), with light green as counterstain. Control smears were treated with saliva.

Mucoproteins.—Toluidine blue method of Lison, as described by Bessis (1956).

Alkaline Phosphatase.—Method of Gomori (1941), using as counterstain neutral red.

Results

Lipids.—The results obtained fell into two distinct classes. In the case of the first, which applied to half of the patients studied, the fat

*This paper is published with the permission of the South African Council for Scientific and Industrial Research, Pretoria.

content of the granulocytes was medium initially (score 202), rose to a peak in the second week, and then declined until the fifth week (Figs. 1, 2, 3, Table I). In the case of the remainder of the patients, there was a high initial fat content (score 243) which fell in the second week. A second peak, lower than the initial one, occurred in the third week. There was then a decline, followed by yet another rise (Fig. 3, Table I).

TABLE I

HISTOCHEMICAL "SCORES" FOR LIPID CONTENT AND ALKALINE PHOSPHATASE AND PEROXIDASE ACTIVITY OF LEUCOCYTES IN KWASHIORKOR PATIENTS AND IN NORMAL CONTROLS

	Controls	Kwashiorkor Patients				
		1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
Lipid ..	225 (180–250)	202[1] (190–230) 243[2] (200–305)	240[1] (210–300) 210[2] (168–225)	225[1] (175–240) 223[2] (170–240)	218[1] (170–230) 192[2] (150–210)	216[1] (165–225) 215[2] (185–230)
Peroxidase	173 (120–198)	228 (200–250)	225 (200–235)	240 (215–285)	244 (215–290)	235 (210–290)
Alkaline phosphatase	169 (110–204)	180 (115–260)	118 (90–145)	110 (85–125)	103 (75–130)	135 (95–175)

[1] Group with initial lipid rise.
[2] Group with initial lipid decline.

When the data shown in Figs. 2 and 3 are combined (Fig. 1) it is seen that the average fat content for all patients is fairly constant over a period of five weeks, with a low peak in the second week and a slight subsequent decline. The average score in the first week was 223. In the controls the granulocyte fat content was either medium or high, both in the case of adult whites and of Bantu children, and remained remarkably constant over a period of five weeks. The average score for the children was 225.

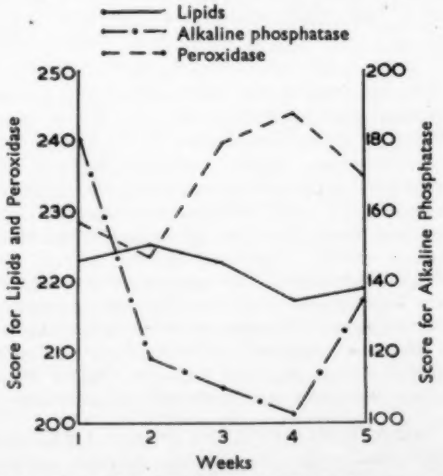

FIG. 1.—Average lipid, peroxidase, and alkaline phosphatase curves of all the patients.

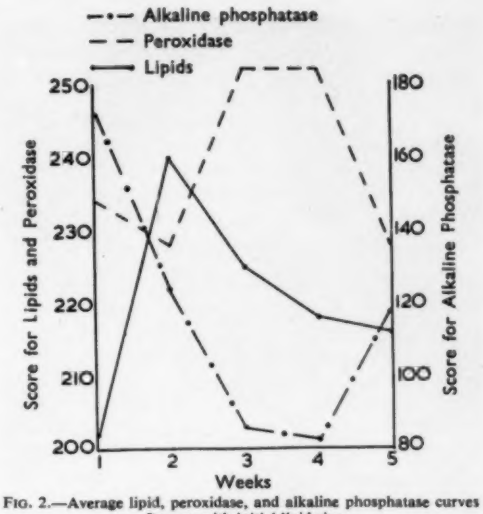

FIG. 2.—Average lipid, peroxidase, and alkaline phosphatase curves of group with initial lipid rise.

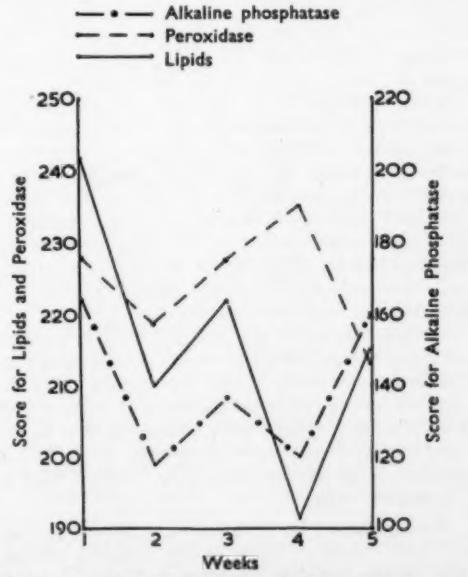

FIG. 3.—Average lipid, peroxidase, and alkaline phosphatase curves of group with initial lipid decline.

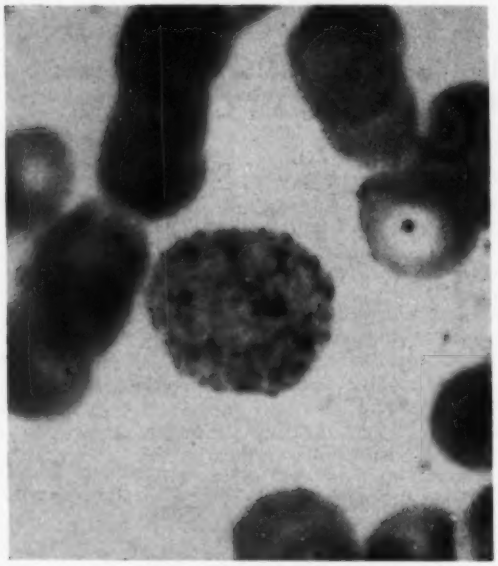

FIG. 4.—Lipid staining of neutrophil of kwashiorkor patient at beginning of treatment. Sudan black B, × 800.

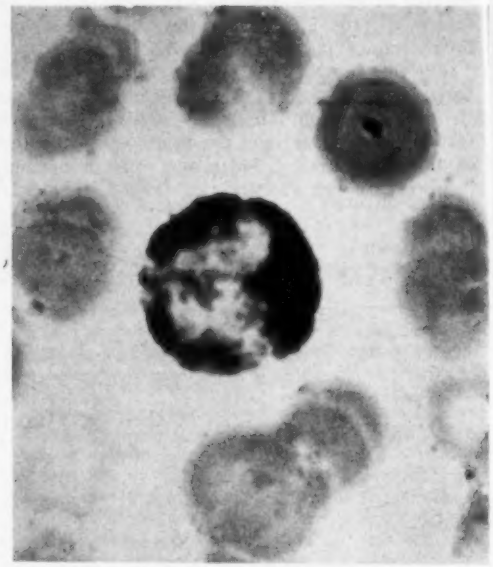

FIG. 5.—Lipid staining of neutrophil of the same kwashiorkor patient as in Fig. 4 after 14 days' treatment. Sudan black B, × 800.

Kinnear and Pretorius (1957) found that in kwashiorkor the total serum lipids are low initially, rise to a peak in a week and then decline. The latter biochemical findings show some analogy to the white cell lipid data of the first type, but not to those of the second type.

Three successive liver biopsies were carried out at intervals of 14 days on each of 12 patients with kwashiorkor, in order to determine the degree of fatty infiltration of the liver. At the second biopsy it was obvious that the fat content of the liver had significantly decreased. The decrease in liver fat content occurs at the same time as the increase in total serum lipids, and is in conformity with the histochemical lipid results of the first type but not with those of the second type.

In order to see if any relation existed between the lipid content and the "age" of granulocytes, Cooke counts were made on smears taken at random during the investigation and stained with Wright's stain. Shifts both to the left and to the right were found to occur at different times, but, in general, more fat was present in "older" than in "younger" cells.

Peroxidase.—Fig. 1 shows that the peroxidase and lipid values are more or less inversely related. This is also roughly the case in Fig. 2. From Fig. 3 it can be seen that initially the curves for fat and peroxidase tend to run parallel, but that the relationship later becomes roughly inverse. In all three figures, the peroxidase values show a high peak in the third and fourth weeks.

In the controls the peroxidase activity was much lower than in the kwashiorkor patients. The average score and range in controls was 173 (120–198) and in kwashiorkor patients 234 (177–310).

Glycogen.—In the majority of the granulocytes glycogen was demonstrated as a diffuse pink coloration in the cytoplasm. A smaller number of granulocytes showed spots throughout the cytoplasm, representing concentrated deposits. In monocytes and lymphocytes, glycogen was dispersed throughout the cytoplasm in granular form.

No differences in colour intensity were observed when comparisons were made between smears at different stages of treatment in the same patient, or between smears obtained from different patients. A comparison between smears from patients and controls also showed no differences.

Nucleic Acids.—Both with DNA and RNA, no differences could be detected between smears taken at different stages of treatment, or between smears from patients and controls.

Mucoproteins.—As in the case of glycogen and nucleic acids, no detectable differences were found at different stages during the disease, or between the intensity of coloration in the nuclei of leucocytes from kwashiorkor patients and controls.

Alkaline Phosphatase.—The activity was found to be fairly high at the start of treatment, with an average score of about 180 (Fig. 1, Table I). During treatment, the level of phosphatase activity dropped sharply, although not to a level which could be considered as low, and then rose again in the fifth week.

For the group of control Bantu children the average score wsa 169 as compared with 130 for the kwashiorkor patients. In the case of the white adults the average score was 48.

Discussion

Although definite figures are given in the case of the results for lipid content and peroxidase and alkaline phosphatase activity, it must be emphasized again that the methods used were of a semi-quantitative nature. No definite conclusions can therefore be reached on the basis of these results alone and certain apparent trends can merely be pointed out.

For the same reason the significance of the findings is difficult to assess at the moment. The conclusions of Wachstein (1955) are, however, of interest. In connexion with the results for lipids he states that significant changes in the lipid staining of leucocytes have not been found under pathological conditions, except in chronic myeloid leukaemia and infectious diseases, where a small proportion may not contain fat. He mentions the fact that sudanophilic granules in myeloid cells increase in number with increasing maturity of the cell.

In the present work, when both types of lipid results are grouped together (Fig. 1), it is seen that the granulocytic fat content remains more or less constant during treatment. There was a slight rise in the second week, when there was a concomitant decrease in the fat content of the liver as revealed by liver biopsies. It therefore seems possible that the granulocyte may serve as a vehicle for the transport of lipids released from the liver during the treatment of kwashiorkor. This possibility obviously requires further investigation, and it is proposed to perform chemical fractionation studies of the lipids of the liver and the leucocytes at different stages during the treatment of kwashiorkor patients.

The fact that in a large number of cases the fat content was high initially, declined, and later rose, seems to indicate that individual factors come into play at different times for reasons which are at present obscure. A factor which may be mentioned is the state of viability of granulocytes, which was revealed by Cooke counts to be subject to temporary variations.

The higher peroxidase activity of the leucocytes in kwashiorkor patients as compared with normal controls is difficult to evaluate. Seabra (1955) found that the peroxidase activity in leucocytes decreased under stress conditions, but high peroxidase activity was noted without exception in all the kwashiorkor patients. Furthermore, the activity showed a tendency to rise still further during treatment and reached a peak in the third to the fourth week.

No detectable differences in staining intensity were found with respect to glycogen, nucleic acids, or mucoproteins. Possibly, with microspectrophotometric methods, some changes might be detected. On the basis of microchemical analyses of liver tissue from infants with manifestations of kwashiorkor, Waterlow (1955) concluded that the RNA in liver is low in relation to DNA and rises during treatment. No difference of this kind was noted in the leucocytes. Waterlow and Weisz (1956) found no change in the glycogen content of the liver in kwashiorkor, an observation which agrees with our histochemical findings on leucocytes.

The fact that the alkaline phosphatase values declined during treatment suggests some degree of similarity between the present results and those of Schwartz (1956) for serum alkaline phosphatase. Certain points of difference, however, must be noted. Schwartz found that serum alkaline phosphatase in kwashiorkor was low at the beginning of treatment, dropped still lower during the first week or two of treatment, and then gradually rose to the level considered normal. In the present work, the initial activity in granulocytes was seen to be fairly close to the average obtained for normal children. It fell, though not to exceptionally low levels, during treatment, to rise once more rather abruptly in the fifth week to almost "normal" levels. It is in the form of the curve, rather than in the degree of phosphatase activity, that some resemblance is seen to the serum results.

Kaplow (1955) and Wiltshaw and Moloney (1955), the latter authors working on separated leucocytes, investigated leucocyte alkaline phosphatase activity in health and disease and

found no relationship between serum alkaline phosphatase and leucocyte alkaline phosphatase activity. Wiltshaw and Moloney concluded that the cellular alkaline phosphatase activity was not regulated *in vitro* by any factor in the serum, but was intrinsic to the leucocyte itself. It is obvious that, before our results for leucocyte alkaline phosphatase activity in kwashiorkor can be evaluated, further studies *in vivo* similar to the above studies of Wiltshaw and Moloney *in vitro* will have to be undertaken.

Summary

Histochemical tests for lipids, glycogen, mucoproteins, alkaline phosphatase, and peroxidase were carried out on blood smears from 20 patients with kwashiorkor and 10 control subjects. In half of the patients studied the lipid content of the leucocytes tended to rise during the first two weeks of treatment, with a slight subsequent decline. In the remainder of the patients there was a drop in the lipid content in the first two weeks. The peroxidase activity of the leucocytes was higher in kwashiorkor patients than in normal controls. With respect to glycogen, mucoproteins, or nucleic acids, no differences were detected by the methods used, either in the same patient at different stages of treatment or between patients and controls. Alkaline phosphatase activity was found to be fairly high at the beginning of treatment, to fall during treatment, and to rise again in the fifth week of treatment. The possible significance of the findings is discussed.

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THE NAGLER REACTION IN PATHOLOGICAL SERA AND ITS USE AS A LIVER FUNCTION TEST

BY

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The alpha toxin of *Cl. welchii* reacts with human serum to produce an opalescence (Nagler, 1939). The nature of the opalescent material is complex, the bulk consisting of lipids and a smaller part of protein (Crook, 1942). This material is derived from lipoprotein complexes, associated with the alpha and beta globulins of the serum, by disruption of their phospholipid components. Purified alpha toxin is a highly specific phosphatase. The action of this phosphatase is directed mainly against lecithin, producing phosphocholine and a diglyceride stearyloleylglyceride (Macfarlane and Knight, 1941). It also acts, but much more slowly, on sphingomyelin to yield phosphocholine and lignoserylsphingosine (Macfarlane, 1942, 1948). The main phospholipid component of human serum is lecithin; the remainder is sphingomyelin and cephalin. These phospholipids are known to be largely metabolized in the liver (Balfour, 1947).

Although the Nagler reaction has been extensively investigated in normal sera, the reaction in sera from pathological states has been neglected. Alterations in phospholipids are common in many diseases, particularly those involving the liver, and might well be expected to influence the result of the Nagler reaction. This paper describes the results of studies of the Nagler reaction in pathological sera, one of the most interesting phenomena being a specific flocculation reaction in parenchymatous liver cell damage.

Materials and Methods

Nagler Reaction.—The *Cl. welchii* alpha toxin was supplied as a dried powder by the Wellcome Research Laboratories, each bottle containing 220 international units. For use, 15.3 mg. dried toxin was freshly dissolved in 10 ml. borate buffer. This gave a concentration of approximately 1.5 international units per ml. A more convenient standard is to use 1 international unit per ml.

Borate Buffer.—Sorenson's at *p*H 7.61 (20°): 475 ml. 0.1 N HCl+525 ml. of borate made up of 12.404 g. boric acid+100 ml. N NaOH per litre distilled water.

0.025 M $CaCl_2$.

Sterile serum was used freshly separated at 37° C. and stored at +4° C.

Alpha Toxin Turbidity and Flocculation Tests.—Three 3 in. × ½ in. test-tubes were placed in a water-bath rack. Into two tubes were measured 0.4 ml. serum+0.1 ml. $CaCl_2$+3 ml. borate buffer+0.5 ml. alpha toxin reagent. The time of addition of the toxin was noted and the rack placed in a water-bath at 37° C. The third tube acted as a control and 0.5 ml. borate buffer replaced 0.5 ml. toxin, the other reagents being the same. Of the two tubes containing toxin, one was left undisturbed and was inspected at intervals for macroscopic flocculation (see below); the other was used for turbidity measurements at the following times from the start of the test: half hour, hourly from one to 12 hours (or beyond 12 hours when possible), 24 hours.

Optical density was recorded in a Hilger absorptiometer using filters red O2 and orange OY2 as for the thymol turbidity. The blank consisted of the control tube, incubated for the same period of time as the test.

Phospholipid in Serum.—Total phospholipid was estimated by the method of Zilversmit and Davis (1950).

Thymol Turbidity and Zinc Turbidity.—The standard technique was employed, using barium sulphate standard (Shank and Hoagland, 1946). When measuring the zinc and thymol turbidities on solutions used in the Nagler reaction 1 ml. of toxin-treated or control serum was mixed with 5 ml. of zinc or thymol reagent and the resulting turbidities noted after the standard period of 30 minutes. In the toxin-treated sera the alpha toxin-flocculated material was first removed by repeated filtration through No. 1 Whatman paper.

Results

Two distinct phenomena resulted from the action of *Cl. welchii* alpha toxin on human sera. The first reaction, common to all types of sera, both normal and from pathological conditions, was the development of a uniform turbidity, as

originally described by Nagler. The second and distinct reaction was aggregation of the fine particles responsible for this turbidity into coarse floccules. These were visible to the naked eye and sedimented into a fluffy deposit on standing. This second reaction of flocculation was limited almost entirely to sera from cases of hepatocellular damage, and so exhibited a certain specificity. The two reactions are analysed separately.

The Turbidity Reaction.—The degree of turbidity developing in alpha-toxin-treated sera was studied over a period of 24 hours. Observations were usually made hourly in the first half of this period, but were not taken during the night, i.e., 12–24 hours. A final reading was taken at the end of the 24-hour period. The development of the turbidity in all cases followed a smooth hyperbolic curve as might be expected in an enzymic reaction. The 24-hour readings were used for comparison, as the reaction was virtually complete in the majority of sera by that interval. Total phospholipid estimations (Zilversmit and Davis, 1950) were done in parallel on each serum and compared with the degree of turbidity. This comparison is shown in Fig. 1 in 150 sera from 10 normal controls and 104 patients suffering from liver disease, namely, infectious hepatitis 12, serum hepatitis three, glandular fever hepatitis four, subacute hepatitis and cirrhosis of the liver 18, alcoholic cirrhosis three, obstructive jaundice (extrahepatic) 10, chlorpromazine jaundice two, phosphorus jaundice one, biliary cirrhosis four, jaundice and/or hepatomegaly of doubtful origin two; from heart disease with hepatomegaly seven; and from heart disease without hepatomegaly three, type II nephritis two, diabetes mellitus two, hypercholesterolaemic xanthomatosis three, idiopathic hyperlipaemia two, xanthomatosis one, myxoedema one, thyrotoxicosis one, sarcoidosis one, chylous ascites one, idiopathic steatorrhoea two, lymphosarcoma one, osteomyelitis one, thrombocytopenic purpura one, myelomatosis 17.

In nearly all sera there is a significant correlation between the total phospholipid concentration

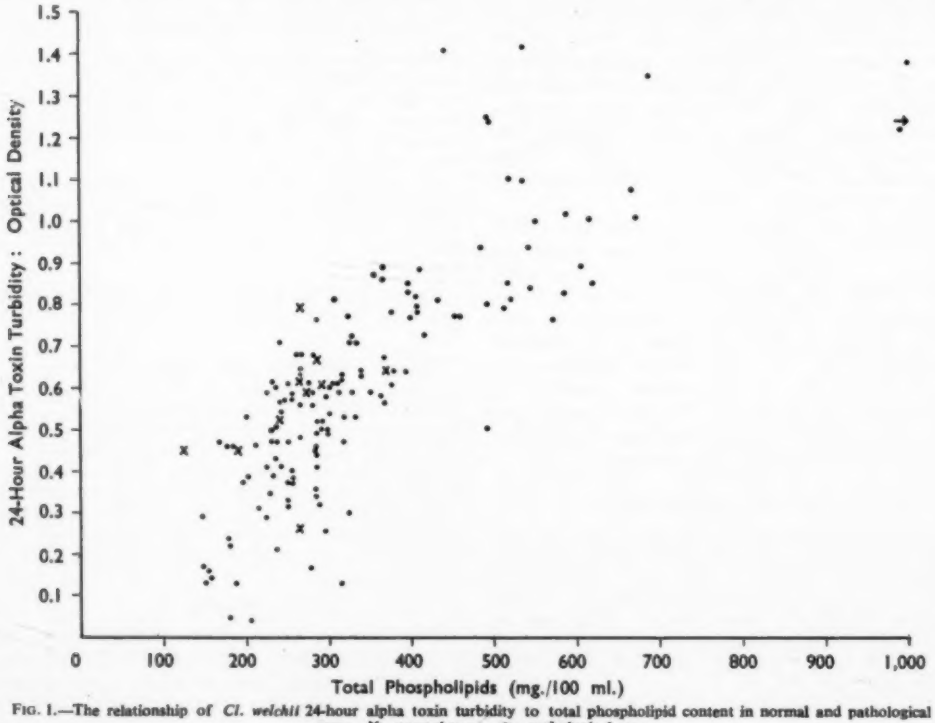

FIG. 1.—The relationship of *Cl. welchii* 24-hour alpha toxin turbidity to total phospholipid content in normal and pathological sera. X = normal sera. ● = pathological sera.

and the degree of turbidity developed in the alpha toxin reaction. Some of the scatter in the results is almost certainly due to phospholipids other than lecithin being included in the total phospholipid figure, but not in the more specific lecithinase reaction. The widest scatter is apparent in sera with very high or very low levels of phospholipid. The group above an optical density of 1.2 are almost entirely derived from cases of xanthomatosis, either hypercholesterolaemia or idiopathic hyperlipaemia. This may represent a significant deviation in these disorders, but too few cases have been studied for a valid analysis. The contrary group of sera with an optical density below 0.25 are nearly all from cases of multiple myelomatosis. Results from 17 cases of multiple myelomatosis are shown separately in Fig. 2 and compared with nine normals. All values for cases of myelomatosis save one lie below the line AB in Fig. 2. Of all human sera examined, both normal and from miscellaneous pathological states, only 12 showed an optical density below 0.25 in the 24-hour toxin turbidity test (Fig. 1). Of these, nine were cases of multiple myelomatosis (approximately 50% of all cases of multiple myelomatosis). The other three sera showing these exceptionally low figures had liver disease. Two were long-standing and severe cases of cirrhosis, the third a case of transient jaundice, thought to be infectious hepatitis.

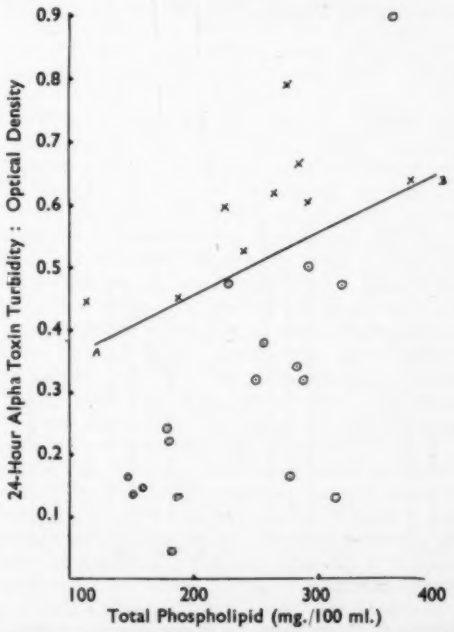

FIG. 2.—The relationship of *Cl. welchii* 24-hour alpha toxin turbidity to total phospholipid content in nine normal and 17 myelomatosis sera.

As was expected, high toxin turbidity values were found in conditions where lipoproteins are known to be increased. These were any type of obstructive jaundice, extrahepatic or intrahepatic, including the obstructive phase of infectious hepatitis, biliary cirrhosis, idiopathic hyperlipaemia, hypercholesterolaemic xanthomatosis, type II nephritis, and diabetes mellitus. Low values were found in severe cirrhosis of the liver or liver failure, idiopathic steatorrhoea, chylous ascites, and, as already described, in multiple myelomatosis.

The Flocculation Reaction.—While studying the Nagler alpha toxin reaction in sera from pathological conditions, it was noticed that certain of these sera exhibited a coarse, macroscopic flocculation of the liberated particles. This flocculation was entirely independent of the total phospholipid content of the serum, and could occur at high, normal, or low lipid levels. The usual diffuse turbidity reaction always preceded the flocculation in all cases, but the time of flocculation from the initiation of the reaction varied considerably. No flocculation occurred in any of 10 normal sera during a 24-hour period of observation.

This flocculation reaction appeared on further analysis to be highly specific for sera from cases of liver disease, characterized by hepatocellular damage. Thus, in 10 out of 11 cases of infectious hepatitis in an active phase following the onset of jaundice, flocculation occurred within a 12-hour period; Case 2 flocculated within 24 hours. Further, the time of flocculation was shortest in the acute phase of the illness and progressively lengthened and recovery ensued (Fig. 3). Similarly flocculation occurred in six sera from three cases of hepatitis in glandular fever, and in four sera from two cases of serum jaundice, all in the period of active cellular damage. Sera from one mild case of glandular fever hepatitis with minimal, transient jaundice, and one case of serum jaundice in the recovery phase, exhibited no flocculation. Flocculation observed at 24 hours represented an unknown time reading between the last recorded reading on the previous evening and the final 24-hour morning reading. This interval is represented by the shaded areas in Fig. 3. Thus in the final stages of recovery of Cases 1 and 2

flocculation occurred at an unknown time between 12 and 24 hours.

Out of 21 cases of subacute hepatitis or cirrhosis, 12 sera from 11 cases gave a positive flocculation reaction before 12 hours and two sera from two further cases flocculated between 12 and 24 hours. The aetiology of the majority of these cases was obscure, as is usual in developed cirrhosis, but three were preceded by a long alcoholic history, one was associated with a granuloma of doubtful aetiology, possibly sarcoid, and one with extensive secondary carcinomatosis and necrosis. Sera from all 13 cases giving a positive alpha toxin flocculation reaction had raised thymol and zinc turbidity values. In the remaining sera from eight other cases no alpha toxin flocculation occurred. Of these eight negative cases, four had normal thymol turbidity and zinc sulphate turbidity reactions, but the other four had raised values.

As opposed to disease in which hepatocellular damage predominates, in obstructive jaundice of varying aetiology, no flocculation occurred in any sera, although total phospholipid content was usually increased as already indicated. Thus no flocculation was observed in 10 cases of extra-hepatic obstruction due to gall-stones and carcinoma, two cases of intrahepatic obstruction due to chlorpromazine, one due to phosphorus poisoning, one case of congenital atresia of the bile ducts with biliary cirrhosis, and three out of four adult cases of biliary cirrhosis in the obstructive phase. One case of biliary cirrhosis did flocculate at 13 hours. The significance of this is discussed later.

In seven cases of liver disease, with palpable enlargement due to chronic heart failure, two gave a positive flocculation reaction only in the 12–24-hour period, one at 13 hours, the other at 24 hours. Both cases had normal thymol and zinc turbidity reactions.

Two other cases of liver disease, one jaundiced and one with a palpable liver but not jaundiced, were included in this series, although of doubtful aetiology. Neither showed alpha toxin flocculation.

As controls, in addition to sera from cases of obstructive jaundice, a number of sera from 10 normal people, male and female, 17 cases of myelomatosis, and 22 miscellaneous pathological conditions, as listed in the description of the turbidity reaction, were studied for the flocculation reaction.

None of the control series showed any flocculation in the first 12 hours of the turbidity

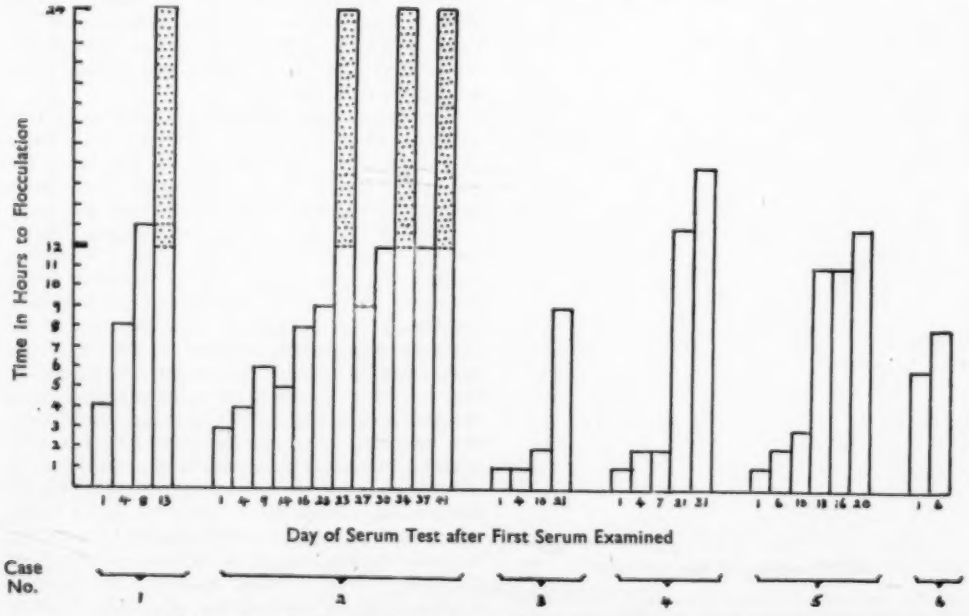

FIG. 3.—*Cl. welchii* alpha toxin flocculation reaction in six cases of acute infectious hepatitis.

tests nor did any normal control at the end of 24 hours. Two cases of steatorrhoea, one case of mild heart failure without a palpable liver, and the case of thrombocytopenic purpura with a palpable liver did show flocculation between 12 and 24 hours. In none of these four cases, with the possible exception of the thrombocytopenic patient, could any evidence be found for a pathological lesion of the liver. The thrombocytopenic patient proved in the end to have a diffuse lipoid infiltration of the spleen and liver, without lipaemia, of unidentified origin. It would appear that flocculation up to 12 hours is characterized by hepatocellular damage; flocculation between 12 and 24 hours is less specific and may occur in occasional sera from pathological cases without liver disease, but is obviously rare, and did not occur in the 10 normal controls. If all cases showing flocculation in the 12–24-hour period are excluded, then no false positives are found in any control sera, and four cases are excluded from the total of all liver disease cases showing flocculation.

Role of Phospholipid in Thymol and Zinc Sulphate Turbidity Tests of Liver Function.—Both the thymol turbidity and the zinc sulphate turbidity tests are dependent on a complex of protein factors. Turbidity is promoted in both by a high gamma globulin concentration, which forms the bulk of the precipitate (Marrack, Johns, and Hoch, 1950) and a low albumin concentration (Maclagan, Martin, and Lunnon, 1952). The zinc sulphate turbidity is more closely correlated with the globulin content of the serum, whereas the thymol turbidity is partly controlled by the lipoprotein concentration (Maclagan and Bunn, 1947; Cohen and Thompson, 1947). As alpha toxin disrupts the major lipoprotein constituents of human serum, the effect on these two turbidity tests should be a measure of their dependence on lipoprotein. The thymol and zinc sulphate turbidities were therefore measured on a number of abnormal sera, where these values were raised in association with liver disease, and compared with values measured after the sera had been exposed to toxin. Results are shown in Table I. All thymol turbidity values were significantly lower, in the majority of cases by 30 to 65% after lipoprotein disruption by the alpha toxin. The zinc turbidity by contrast showed a variable response. In some cases the turbidity was reduced, but never to a comparable degree as in the thymol reaction; in some cases there was little change; while in a number there was a definite increase in the turbidity. In the latter sera destruction of lipoprotein would seem to expose the gamma globulin to the zinc sulphate action with a corresponding increase in metal-protein precipitate.

TABLE I

CHANGES IN THYMOL AND ZINC TURBIDITY VALUES AFTER TREATMENT OF SERA BY *CL. WELCHII* ALPHA TOXIN

RESULTS WITH NAGLER REACTION

Case	Day of Specimen	Thymol Turbidity Units			Zinc Turbidity Units			
		Control Serum	Alpha Toxin-treated Serum	% Change —	Control Serum	Alpha Toxin-treated Serum	% Change +	% Change −
1		5·7	3·7	35·0	9·8	6·8		30·5
2	1	7·4	4·1	44·5	8·8	6·6		25·0
	4	7·8	3·3	57·5	6·3	6·1		3·0
	9	4·3	2·5	42·0	7·4	5·3		28·0
	14	5·3	2·7	49·0	5·7	6·3	10·5	
	16	4·7	3·7	21·0	5·7	5·7	No	change
	20	5·5	2·9	47·0	5·7	7·0	23·0	
	23	4·1	2·5	39·0	6·1	5·3		13·0
	27	5·5	3·7	32·5	6·1	8·4	38·0	
	30	4·9	2·9	41·0	5·7	7·0	23·0	
	34	5·3	2·5	53·0	6·1	6·6	8·0	
	37	4·9	2·5	49·0	7·0	5·7		18·5
	41	5·7	2·5	56·0	5·9	5·7		3·5
3	1	8·2	3·7	55·0	6·8	7·4	9·0	
	4	8·6	3·3	61·5	7·8	7·6		2·5
	10	9·0	4·5	50·0	7·8	9·6	2·0	
	21	7·0	3·7	47·0	6·1	8·8	44·0	
5	1	13·9	4·9	65·0	15·2	8·6		43·5
	6	10·0	4·9	51·0	11·5	9·0		22·0
	10	7·8	4·1	47·5	10·2	8·2		19·5
	16	6·1	4·1	33·0	7·8	7·4		5·0
	20	4·3	3·3	23·0	7·8	7·8	No	change
10	1	14·8	7·0	53·0	12·7	13·1	3·0	
	2	16·4	7·0	57·5	9·4	9·0		4·0
14	1	9·8	7·8	20·0	9·8	13·5	38·0	
	10	13·5	9·0	33·5	11·9	14·8	24·0	
15	1	9·4	5·3	42·5	12·3	9·0		27·0
	9	8·2	4·5	45·0	11·5	7·4		35·5
16	1	7·4	6·6	11·0	7·0	9·8	40·0	
	21	6·6	6·1	7·5	8·2	9·4	15·0	
21		14·1	5·7	60·0	23·0	13·9		39·5
23		13·9	6·1	66·0	25·0	19·0		24·0
25		9·4	4·9	48·0	10·7	10·9	2·0	
27		14·3	8·2	29·0	27·5	21·3		22·5
28		4·5	2·5	44·5	12·7	12·3		3·0
29		4·5	3·1	31·0	9·0	9·8	9·0	
30		23·4	4·7	8·0	18·0	14·8		17·5

Discussion

The phospholipids of human serum exist as complexes with protein, particularly the alpha and beta globulins. In normal serum examined by zone electrophoresis the lipid staining beta component of these lipoproteins is located in the beta globulin region, while the alpha component lies between the albumin and the alpha 1 band (Dangerfield and Smith, 1955; Kunkel and Slater, 1952). Distribution and density of radioactive phosphorus incorporated in these complexes closely follow the fat-staining components (Dangerfield and Smith, 1955). The exact mode of linkage of lipid and protein remains obscure. The complex is probably a micelle consisting of a nucleus of phospholipid, the polar groups centrifugally orientated, surrounded by a shell of partly unfolded protein molecules applied to the

phospholipid. The main phospholipid in human serum is lecithin, accompanied by smaller quantities of sphingomyelin and cephalin. Also centrally integrated in the fat micelle are cholesterol and some neutral fat. The alpha toxin of *Cl. welchii* disrupts this complex by the action of phosphatase C. Phosphatase C acts specifically on lecithin at the phosphate bond, to yield phosphocholine and diglyceride such as stearyl-oleylglyceride (Macfarlane and Knight, 1941). It has a similar but much slower action on sphingomyelin, yielding phosphocholine and lignoserylsphingosine (Macfarlane, 1942, 1948). The whole lipoprotein micelle is consequently fragmented with the liberation of all its component lipids and protein to give the characteristic Nagler turbidity reaction. The released material has been analysed by Crook (1942), and shown to consist of approximately 30% protein and 70% fat components, including phospholipid, neutral fat, cholesterol, and fatty acids; small traces of sugar were also present. Petermann (1946) by ultracentrifugation demonstrated the disappearance of the X component of human plasma following this disruption. The X component is largely composed of beta globulin. The author has confirmed by paper zone electrophoresis that all lipid-staining material is removed from alpha-toxin-treated sera, in both the alpha and beta regions, although the protein pattern is not appreciably altered. The proportion of protein liberated in the Nagler reaction is comparatively small compared with the total serum globulin.

The variables likely to modify the Nagler reaction include total lipoprotein content of serum, the proportion of alpha and beta lipoproteins, and disturbance of liver cell function. The liver is accepted as being the main source of phospholipid synthesis, apart from the intestinal epithelium. Balfour (1947) has suggested, on evidence furnished by studies on the incorporation of radioactive phosphorus in lipoproteins, that all hyperphospholipaemic states, irrespective of the aetiology, are a result of increased synthesis in the liver and not accumulation in the blood stream. Such states include obstructive jaundice, biliary cirrhosis, nephrosis, idiopathic hyperlipaemia, and hypercholesterolaemic xanthomatosis. If confirmed, his conclusion is surprising, since in a condition such as acute virus hepatitis obstruction is accompanied by widespread hepatocellular damage. Individual variations in the alpha and beta components of the lipoproteins in the above conditions have been described by Kunkel and Slater (1952) and Dangerfield and Smith (1955). In nearly all hyperphospholipaemic sera the beta component was increased. The alpha component was reduced or absent in obstructive jaundice, infective hepatitis in the acute stage, and nephrosis. In diabetes, myxoedema, and xanthomatosis, the alpha component remained. An additional component, the pre-beta lipid, also appeared in certain pathological sera. Ultracentrifuge studies have revealed similar changes. Thus Pierce, Kimmel, and Burns (1954) reported an increase in components Sf 0-12, 12-20, and 20-100 and a decrease in Sf 100-400 in plasma in acute hepatitis. The study of variations in individual phospholipids has been hampered by the inadequacy of chemical methods available. However, Petersen (1953) found in acute virus hepatitis that the hyperphospholipaemia was due to lecithin components, sphingomyelin and cephalin remaining remarkably constant. In severe liver failure all phospholipids were decreased.

In studying the Nagler reaction, sera from a wide range of different pathological states were sampled, in which lipoproteins might be expected to vary from normal, either in quantity, quality, or relative proportions, and particularly as a result of altered liver metabolism.

The degree of turbidity in the alpha toxin turbidity reaction was roughly proportional to the total phospholipid content of sera (Fig. 1). This correlation held for both normal sera and those from a wide range of pathological conditions. The alpha toxin turbidity presumably parallels lecithin content rather than total phospholipid content. This must account for some of the scatter of the results shown in Fig. 1. Cephalin will contribute to the total phospholipid result but probably not to the Nagler reaction. An increased toxin turbidity ran parallel with increased phospholipids, particularly in obstructive jaundice, biliary cirrhosis, nephrosis, diabetes, idiopathic hyperlipaemia, and hypercholesterolaemic xanthomatosis. The decrease in alpha toxin turbidity in multiple myelomatosis was sometimes, but not necessarily, accompanied by a parallel decrease in the total phospholipid value. Although not exclusive to multiple myelomatosis, such a striking decrease was of diagnostic value. Low values occasionally occurred in liver failure. One possible explanation for the poor reaction in myeloma serum is the presence of a shell of abnormal proteins preventing the penetration of alpha toxin to the nucleus of lecithin in the fat micelles.

The result of greatest interest was the specific flocculation reaction encountered in liver disease accompanied by parenchymatous cellular damage. Flocculation was entirely independent of the absolute phospholipid content, measured by either alpha toxin turbidity or total phosphorus. Flocculation within 12 hours of the start of the reaction was considered significant, and between 12 and 24 hours less specific, although not occurring in normal sera. Flocculation was constant in all cases of acute virus hepatitis, serum jaundice, and glandular fever hepatitis in the active stages of the disease. The time taken to flocculate increased progressively with recovery (Fig. 3). In chronic hepatitis and cirrhosis of the liver, over 50% of the cases gave positive alpha toxin flocculation tests. In all these cases the lipid-protein complex released by alpha toxin appeared to be altered in such a way that aggregation of the normally fine particles was encouraged. The obvious explanation for this aggregation was an alteration in the relative proportions of plasma proteins in the surrounding medium, particularly the decrease in albumin and the increase in globulin in liver disease. Flocculation was, however, most constant in acute hepatitis where such an alteration was minimal, and least constant in cirrhosis where the alteration was maximal. Alternatively the liver cell may have a specific metabolic function in linking lipid to protein in the plasma lipoprotein micelle, and this function may be disturbed in extensive liver parenchymatous cell damage. A third possibility is that alteration in the proportion of individual lipoproteins, particularly alpha and beta globulins, may result in predominance of a more flocculable moiety. Changes in alpha and beta lipoproteins in liver disease have already been discussed. An attempt to measure the proportion of saturated to unsaturated fatty acids in lecithin in liver disease was unsuccessful. Long and Maguire (1954) have shown that phospholipase C acts only on lecithins containing unsaturated acids in the C position.

Used as a test of liver function, alpha toxin flocculation only approximately correlated with other turbidity tests of hepatocellular disease. In acute infectious hepatitis, glandular fever hepatitis, and serum hepatitis, correlation was good, the alpha toxin flocculation being positive in the acute phase of hepatocellular damage as judged clinically, and by the thymol turbidity and zinc turbidity tests. With recovery, time to flocculation lengthened progressively to beyond the 24-hour limit. Alpha toxin flocculation returned to normal before thymol turbidity and zinc sulphate turbidity. In cirrhosis and chronic hepatitis correlation was less good. In all cases where alpha toxin flocculation was positive, thymol and zinc turbidities were increased. But in the eight cases where alpha toxin flocculation was negative four had raised thymol and zinc turbidity values. Such a dissociation presumably reflected the different underlying mechanisms of the various flocculation reactions.

Both thymol and zinc turbidity reactions are dependent on a balance between the various plasma proteins. Gamma globulin promotes and albumin inhibits turbidity (Maclagan *et al.*, 1952). Although the bulk of the precipitate in the thymol reaction is gamma globulin (Marrack *et al.*, 1950), the reaction is also dependent on lipoprotein (Maclagan *et al.*, 1952). The zinc reaction, by contrast, correlates more closely with the gamma globulin content of sera in most pathological states (Kunkel, 1947). The degree of lipoprotein dependence of both tests should be roughly assayable by previous exposure of sera to alpha toxin, which disrupts all lipoprotein. A consistent and appreciable diminution of the thymol turbidity by values between 30 and 65% resulted in all the sera examined, including cases of acute hepatitis and cirrhosis of the liver (Table I). The same effect was not apparent in the zinc test. Results after exposure to alpha toxin varied considerably, in some sera actually being increased. The importance of lipoprotein in contributing to the thymol reaction was confirmed. In the zinc reaction its removal had little effect, although in some sera this apparently increased the proportion of metal globulin complex precipitated.

Summary

The Nagler *Cl. welchii* alpha toxin reaction has been studied in 150 sera from 10 normal adults and 102 pathological conditions.

The turbidity developed was approximately proportional to the total lipoprotein in all sera.

The majority of sera from 17 cases of multiple myelomatosis gave a poor reaction which, with an abnormally low turbidity, is useful in differential diagnosis.

In hepatocellular damage a specific flocculation occurred within 12 hours of the start of the reaction which was independent of the total lipoprotein content of the sera.

This flocculation characterized all cases of acute hepatitis, including acute infectious hepatitis, serum jaundice, and glandular fever hepatitis.

Flocculation occurred in over 50% of cases of cirrhosis of the liver.

Flocculation did not occur in obstructive jaundice of either intra- or extrahepatic type, nor in other types of liver disease.

Destruction of lipoprotein in sera by alpha toxin resulted in a diminution of the thymol turbidity but not of the zinc turbidity reaction.

The author is indebted to the medical and technical staff of the clinical laboratories for all their help. In particular Mr. C. Sait took great trouble in the collection of the necessary specimens.

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THE ESTIMATION AND RECOVERY OF DEXTRAN SULPHATES IN BIOLOGICAL FLUIDS

BY

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The dextran sulphates resemble heparin in being active inhibitors of several different forms of biological activity, e.g., blood coagulation, serum complement activity, and certain enzyme systems (Walton, 1954). In each case the activity of the dextran sulphate appears to be dependent upon direct interaction with one or more protein components of the biological system under investigation. In fact, the interaction of certain varieties of dextran sulphates with specific plasma proteins has been utilized as a means of plasma fractionation (Oncley, Walton, and Cornwell, 1957).

Work in this field has made it necessary to seek a simple method of assessing quantitatively the concentration of dextran sulphates of given physical and chemical characteristics in biological fluids and in some instances of recovering the dextran sulphate from these fluids. The general resemblance in chemical constitution and behaviour between the dextran sulphates and heparin suggested a survey of the published methods for the estimation and isolation of heparin to determine whether or not such methods were equally applicable to the estimation of dextran sulphates.

Materials

Dextran Sulphates.—Sulphuric esters of two different dextran fractions were prepared as previously described by Ricketts (1952). The parent dextrans had intrinsic viscosities [η] of 0.04 and 0.67 respectively. The dextran sulphates derived from them contained 16.3% and 16.1% sulphur and their approximate molecular weights were I/7: 7×10^3 and G.A: 2×10^6.

Heparin.—A commercial sample of "pularin" (Evans) was used. This sample contained 8.61% sulphur and assayed at 113 i.u./mg.

Azure A.—Batch No. 005804 of "revector" stain (Hopkins and Williams Ltd.) was used without further purification.

Thromboplastin.—Desiccated rabbit brain powder "bacto-thromboplastin" (Difco Ltd.), and acetone-extracted human brain prepared according to the method of Biggs and Macfarlane (1953) were used as specified.

Pepsin and Trypsin.—As dry powder were supplied by British Drug Houses Ltd.

Other Reagents.—Petroleum ether (B.P. 60–80° C.) was supplied by May and Baker Ltd.; phenol, ammonium sulphate, and sodium chloride were employed as analytical grade reagents.

Citrated Plasma.—Whole human blood was collected from normal volunteers in a paraffined syringe and mixed with 3.8% (w/v) sodium citrate solution in the proportions 9:1. Plasma was separated after centrifugation at 3,000 r.p.m. for 15 minutes.

INTERACTION WITH BASIC DYES

It is well known that sulphated polysaccharides react with certain basic dyes in such a way as to change the normal (orthochromatic) colour of the dye to another (metachromatic) colour. It was shown by MacIntosh (1941) that the metachromatic reaction produced by heparin when added to the basic dye, toluidine blue, could be utilized quantitatively for the estimation of this substance. Walton and Ricketts (1954) showed that the method was applicable to the estimation of dextran sulphates in aqueous solution and investigated the physico-chemical basis of the reaction. It was later found that the thiazine dye azure A, which is chemically closely similar to toluidine blue, could be employed with identical effect and was preferable in that less variation was encountered between different batches of the dye.

Reagents

(i) Azure A Solution.—0.003% (w/v) azure A in 0.01 N hydrochloric acid containing 0.2% (w/v) sodium chloride.

(ii) Sodium Chloride Solution.—0.2% (w/v).

(iii) Dextran Sulphate Solutions. — Aqueous solutions of dextran sulphates 1/7, ranging in concentration from 1 μg. per ml. to 200 mg. per ml., and similar concentrations of the acid polysaccharide in citrated plasma, serum or albumin solution.

Method

The technique used was identical with that described by Walton and Ricketts except that the azure A reagent was substituted for the toluidine blue reagent previously used.

Results

(i) Dextran Sulphate in Aqueous Solution.—In confirmation of the results previously obtained, as illustrated in Fig. 1 it was found that an excellent linear relationship existed between the percentage of

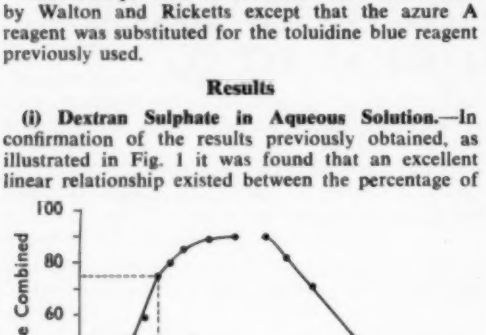

FIG. 1.—Relationship between dextran sulphate concentration and percentage of azure A combined during the production of metachromasia.

the dye bound and the concentration of dextran sulphate over the range 0 to 6 μg./ml. Above this range, increments in concentration of dextran sulphate produced relatively little further increase in the percentage of dye bound till a point was reached at which the latter was virtually constant over the range 12 to 5,000 μg. per ml. Thereafter at still higher

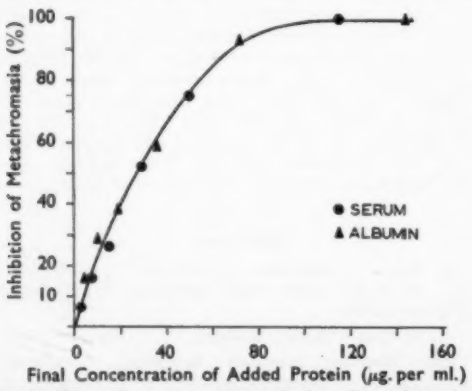

FIG. 2.—Effect of proteins on the production of metachromasia (showing percentage inhibition of metachromasia given by 10 μg. per ml. dextran sulphate under standard conditions by varying concentrations of protein).

concentrations (5 to 200 mg. per ml.) the metachromatic reaction showed progressive inhibition, also in an apparently linear fashion.

(ii) Dextran Sulphates in Presence of Proteins.—It was found that the metachromatic reaction was completely inhibited in plasma. Moreover, on attempting the extraction of the dextran sulphate-dye complex with petroleum ether a stiff viscous gel formed and no extraction was possible. Similar results were obtained when human serum or albumin solutions were substituted for plasma and as little as 0.001% of each protein produced 25% inhibition of the reaction and 0.01% protein completely masked the metachromasia (Fig. 2).

Masking of the metachromasia given by other acidic polysaccharides was similarly found by French and Benditt (1953) and by Hamerman and Schubert (1953).

Evaluation

From these results it was evident that the metachromatic method was only suitable for the estimation of dextran sulphates in "protein-free" solutions, the latter being defined as containing less than 0.0001% protein. In such solutions the method was extremely sensitive, allowing the estimations of concentrations between 1 and 6 μg. per ml. with an error of ±5%. It was necessary to make accurate dilutions of an unknown solution to ensure that values obtained fell upon this linear portion of the curve relating percentage of dye bound to the dextran sulphate concentration. This method has been found useful, for example, in estimating the excretion of dextran sulphates in the urine of experimental animals (Ricketts, Walton, and Saddington, 1954) and in man (Jeavons, Walton, and Ricketts, 1956) where the small amounts of protein normally present are insufficient to interfere with the production of metachromasia.

ATTEMPTED RECOVERY AND ESTIMATION AFTER PROTEIN PRECIPITATION

In view of the difficulties previously encountered, attempts were made to remove the proteins of plasma before the recovery and colorimetric estimation of dextran sulphates by the metachromatic method. Protein precipitation by a number of conventional methods (trichloracetic acid, sodium tungstate, copper tungstate, etc.) gave unsatisfactory results. Ammonium sulphate and phenol precipitations proved to be more satisfactory and are described in detail.

Precipitation of Proteins with Ammonium Sulphate

Principle.—Bassiouni (1954) used ammonium sulphate for the isolation and estimation of heparin in tissue fluids, but obtained recoveries of 50% only. Attention was therefore paid to the effects of variation of *p*H, temperature, and ammonium sulphate concentration to ascertain whether or not better recoveries could be obtained.

Reagents.—These are as follows :
Ammonium sulphate (Analar grade)
2 N sodium hydroxide solution
0.1 N hydrochloric acid
0.2% (w/v) sodium chloride solution
Buffer Reagents. — 0.2 M disodium hydrogen phosphate and 0.1 M citric acid

Method.—Plasma containing various amounts of dextran sulphate was added in 2.2 ml. volumes to 4.8 ml. of 0.2% NaCl and 0.5 ml. 2 N NaOH, and 6.5 g. ammonium sulphate crystals was added, the whole shaken for three minutes, allowed to stand, and then filtered. An aliquot of the filtrate was titrated against 0.1 N HCl and the amount of acid required to neutralize the bulk of the filtrate calculated. The dextran sulphate was then estimated in the neutralized filtrate by the colorimetric method of MacIntosh. The process was repeated at a range of *p*H values from 3.75 to 12.5 (using 5.3 ml. volumes of buffer in place of 4.8 ml. NaCl and 0.5 ml. 2 N NaOH) and varying concentrations of ammonium sulphate. Protein concentrations in the various filtrates were estimated from values obtained for the optical density at 2,800 Å using a " unicam " spectrophotometer.

Results.—Small concentrations of residual protein interfered with the subsequent metachromatic reaction as expected, so that saturation with ammonium sulphate was necessary. Variations in *p*H and temperature did not significantly influence recoveries which varied from 40 to 60% of known added amounts of dextran sulphate. These results are summarized in Tables Ia and Ib.

TABLE Ia
INFLUENCE OF AMMONIUM SULPHATE CONCENTRATION ON RECOVERY OF DEXTRAN SULPHATE

Percentage saturation ammonium sulphate	33	66	100	100
Percentage protein removal	34	86	95	95
,, dextran sulphate recovery	Nil	Nil	50	54·2

TABLE Ib
RECOVERIES OF DEXTRAN SULPHATE ADDED TO PLASMA (AMMONIUM SULPHATE METHOD)

*p*H	Concentration of Added Dextran Sulphate (mg.%)	Concentration Found (mg.%)		Percentage Recovery	
		(a)	(b)	(a)	(b)
12·5	60	24·0	26·4	40	44
	30	13·1	12·0	43·7	40
	15	6·6	8·6	44·2	57
	7·5	3·8	4·2	50	56
7·0	40	21·7		54·2	
	7·5	3·8		50·0	

(a) and (b) are results obtained in duplicate estimations.

Precipitation with Phenol

Principle.—Phenol added in suitable proportions to plasma produces an initial precipitation of protein which is subsequently redissolved and after standing for several hours the mixture separates into two phases, the lower containing the protein. Monkhouse and Jaques (1950) applied this property of phenol to the separation of heparin from proteins and obtained 80 to 90% recoveries of heparin added to whole blood. This principle was therefore applied to the problem of separating dextran sulphates from plasma.

Reagents.—The following were used :
A phenol solution of 800 ml. melted crystals made up to 1 litre with water
Dextran sulphate (1/7) dissolved in human plasma in concentrations from 3.75 mg. to 200 mg.%
Ether and 0.85% saline (w/v)

Method.—Dextran sulphate in plasma was added in 5 ml. volumes to 5.5 ml. volumes of phenol solution and allowed to stand 10 to 12 hours after vigorous mixing. The tubes were then centrifuged and the clear upper layer pipetted off. The remaining phenol layer was washed with saline and the washings added to the aqueous layer. The combined aqueous phase was washed with 5 ml. ether which was then pipetted off. The residual ether was evaporated by heating at 65° C. for a few minutes on a waterbath. The dextran sulphate in the aqueous phase thus obtained was estimated colorimetrically by the metachromatic reaction.

Results.—The dextran sulphate separated from protein in the aqueous phase and estimated colorimetrically gave values indicating recoveries of approximately 75 to 95% of added dextran sulphate. Examples of recoveries at various dextran sulphate concentrations dissolved in plasma are given in Table II. Duplicate results were reproducible with an error of ±5%.

TABLE II
RECOVERY OF DEXTRAN SULPHATE FROM PLASMA (PHENOL METHOD)

Concentration added dextran sulphate (mg.%)	200	100	50	60	60	30	15	7·5	3·75
Concentration found (mg.%)	195·2	93·6	46·5	55·8	53·4	25·5	11·5	5·7	3·2
Percentage "recovery"	97·6	93·6	93·0	93·0	89·0	85·0	76·7	76·5	85·0

Evaluation of Protein Precipitation.—Ammonium sulphate precipitation was less time-consuming than phenol precipitation but had the obvious disadvantage of low recoveries of dextran sulphate added to plasma and interference with the metachromatic reaction unless protein precipitation was virtually complete. The phenol method gave good and reproducible recoveries and there was no interference subsequently with the metachromatic reaction. Attempts to reduce the time of standing allowed in this method to periods varying between one and six hours gave unsatisfactory recoveries of from 45 to 75%.

ATTEMPTED PRELIMINARY DIGESTION OF PROTEIN

Principle

Harington, Pochin, and Squire (1940) reported the prevention of protein interference during the estimation of Evans blue in plasma by preliminary digestion with pepsin. In the present context digestion was attempted with pepsin and trypsin.

Reagents

The following were used:
Pepsin, 5% (w/v) aqueous solution
Trypsin, 5% (w/v) aqueous solution
Hydrochloric acid, 5 N
Sodium hydroxide, 5 N
Dextran sulphate I/7 dissolved in plasma
Sodium chloride solution, 0.2% (w/v)

Method

Pepsin solution, 0.4 ml., was added to 2 ml. plasma to which dextran sulphate had been added in concentrations from 2 to 60 mg.%. The *p*H was adjusted to 1.7 by the addition of 0.20 ml. 5 N HCl, before incubation for three hours at 40° C. Then 0.2 ml. 5 N NaOH was added to bring the *p*H to 3.5 and the volume was made up to 20 ml. using 0.2% NaCl. Aliquots were used for the colorimetric estimation of dextran sulphate using the metachromatic reaction.

Results

Protein digestion did not occur in the presence of dextran sulphate, whereas in control plasma samples free from dextran sulphate it proceeded satisfactorily. Dextran sulphate was not detected subsequently by the metachromatic reaction. Trypsin was used at *p*H 8.5 and again no digestion occurred.

Evaluation

Clearly, preliminary digestion of protein with pepsin or trypsin is unsatisfactory as these enzymes appear to be inhibited by dextran sulphate. This was confirmed by the observation that dextran sulphate produced a diminution of the area of haemoglobin denaturation by trypsin on a blood agar plate. Dextran sulphate therefore resembles heparin, which was shown to inhibit tryptic digestion by Horwitt (1940). Papain was found to be equally unsatisfactory for the same reason. Furthermore, using azo-albumin as substrate (Tomarelli, Charney, and Harding, 1949), it was found that dextran sulphate inhibited the proteolytic activities of crystalline preparations of trypsin and chymotrypsin.

ESTIMATION OF DEXTRAN SULPHATE IN PLASMA BY VIRTUE OF ITS ANTICOMPLEMENTARY ACTIVITY

Heparin and other highly charged macromolecular compounds are known to exert an anticomplementary effect in a haemolytic system (Ecker and Gross, 1929). Walton, Ellis, and Taylor (1957) have described a method for the determination of the anticomplementary activity of heparin and dextran sulphates. Attempts to utilize this technique for the estimation of dextran sulphate were satisfactory for saline solutions but for solutions in plasma unreliable results were obtained, due presumably to the occasional anticomplementary activity of plasma and interference by citrate ions.

ESTIMATION OF DEXTRAN SULPHATE BY VIRTUE OF ITS ANTITHROMBOPLASTIC ACTIVITY

Principle

It was known that dextran sulphate inhibited "thromboplastin activity" and recently Grasset and Schwartz (1955) described a method of assay for heparin and dextran sulphate based upon this fact.

Reagents

The following were used:
Fresh citrated human plasma
Thromboplastin suspension in saline
Calcium chloride solution M/40
Dextran sulphate solutions I/7 and G.A in graded concentrations dissolved in saline, serum, or plasma

Method

Plasma, 0.1 ml., and 0.1 ml. of thromboplastin were added to 0.2 ml. of dextran sulphate solution and allowed to stand for 10 minutes at room temperature. Then 0.1 ml. M/40 $CaCl_2$ was added on a water-bath at 37° C. and the clotting time recorded. Triplicate estimations were made for each concentration of dextran sulphate.

Standard curves were constructed relating the clotting times obtained to the corresponding dextran sulphate concentrations. Values for unknown samples were obtained by reference to these curves.

Results

The low molecular weight dextran sulphate (I) gave an apparent linear relationship between clotting times and concentration of added material (Fig. 3). In order to test the validity and reproducibility of this, three runs were made in triplicate over the range 0 to 10 μg. per 0.2 ml. added dextran sulphate solution, using the same substrate plasma and the same batch of thromboplastin. Analysis of the data obtained revealed a significant regression coefficient in each case with no significant difference between the slopes of individual runs. Ninety-five per cent. confidence

TABLE III
REPRODUCIBILITY OF ESTIMATION OF DEXTRAN SULPHATE BY THROMBOPLASTIN METHOD

	Run 1	Run 2	Run 3	Common Regression
Regression coefficient (r)* ..	0·969	0·972	0·951	0·959
Slope (b)*	0·50	0·54	0·60	0·54
Degrees freedom (n–2)	13	13	13	43
Significance (P) ..	>0·001	>0·001	>0·001	>0·001

* Derived from regression lines relating clotting time to concentration of added dextran sulphate.

limits were calculated for the common regression line and the experimental points, with few exceptions, fell within these limits (Table III and Fig. 4). Results varied when different batches of thromboplastin were used and it was necessary to construct a standard curve for any given set of reagents.

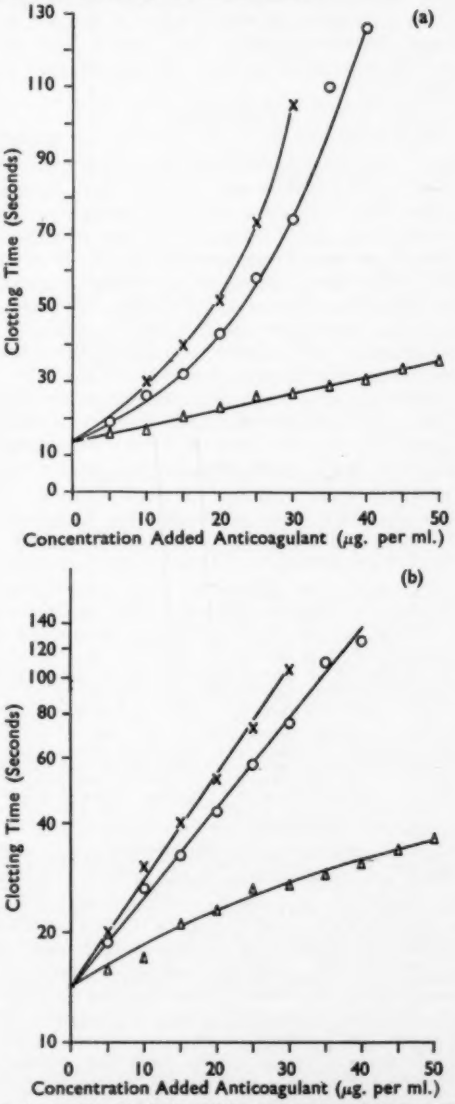

FIG. 3.—Antithromboplastic activity of heparin and dextran sulphates. (Note the use of logarithmic spacing on ordinate in 3b.) X—X heparin, O—O dextran sulphate (G.A), Δ—Δ dextran sulphate (I).

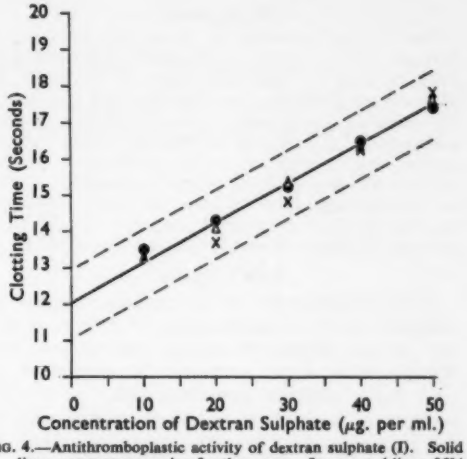

FIG. 4.—Antithromboplastic activity of dextran sulphate (I). Solid line: common regression for three runs. Interrupted line: 95% confidence limits for common regression. ●, Δ, and X: mean experimental points for runs 1, 2, and 3 respectively.

In the case of the large molecular weight dextran sulphate (G.A) and heparin, a curvilinear relationship was obtained (Fig. 3). This could be transformed to a linear one by plotting the logarithm clotting time against concentration of added material (Fig. 3), thus facilitating the assessment of test solution values by reference to the standard curves.

It was observed that when very low (less than 1 μg. per 0.2 ml.) concentrations of dextran sulphate were added to the system there was an apparent pro-thromboplastic effect in that there was a shortening of the base line clotting time. From these observations it was apparent that for the estimation of unknown concentrations of dextran sulphate in aqueous or protein solutions it was necessary to make several dilutions in saline to ensure a final concentration within the range of 2 to 10 μg. per 0.2 ml. of added solution. In practice it was found to be convenient to take the mean of the values obtained for two consecutive dilutions which fell within this range. The results of estimations of various concentrations of dextran sulphate (I/7) in the presence of protein are illustrated in Table IV.

With concentrations less than 0.004% of dextran sulphate in plasma unreliable results are obtained as the added neat or only slightly diluted plasma upsets the concentration of citrate ions, fibrinogen, prothrombin, and accessory clotting factors in the system.

TABLE IV

PERCENTAGE ERROR OF DEXTRAN SULPHATE ESTIMATION BY THROMBOPLASTIN METHOD

Concentration added dextran sulphate (mg.%)	200	200	100	50	25	10	5
Estimated concentration (mg.%) ..	212·5	200	90	54	29	9·5	4·5
Error percentage ..	+6	0	−10	+8	+16	−5	−10

Evaluation

The method provides a reasonable estimate of dextran sulphate in aqueous solution detecting as little as 0.0005% and requiring only 0.6 ml. for triplicate observations. In the presence of plasma the method is still valid down to 0.004% dextran sulphate. It is primarily a method for the estimation of dextran sulphate in the presence of protein and no recovery of the material is possible. It has the disadvantage that estimates at various dilutions may be necessary in order to obtain a concentration somewhere within the range 2 to 10 μg. per 0.2 ml.

DISCUSSION

The estimation of acidic polysaccharides in "protein-free" solution presents little difficulty and can be undertaken readily using MacIntosh's (1941) method which measures the proportion of basic dye bound with polysaccharide. As already mentioned, the method is therefore applicable to the estimation of dextran sulphate in urine. In the presence of proteins, however, the intensity of the metachromatic reaction may be masked partly or completely on account of dye-protein competition. Furthermore, the protein interferes with the separation of the residual dye from the metachromatic complex on addition of petroleum ether, as a stiff viscous gel is produced.

Prevention of the protein interference by preliminary enzymic digestion was not possible, but the interesting fact was revealed that dextran sulphates inhibited the activities of trypsin and pepsin and thus resembled heparin (Horwett, 1940). Attempts to separate the dextran sulphates from protein before estimation in aqueous solution by preliminary precipitation of the proteins revealed that the usual protein precipitants such as trichloracetic acid were valueless. However, ammonium sulphate precipitation gave approximately 50% and the phenol method approximately 95% recoveries respectively, of known amounts of dextran sulphate added to plasma. The latter method had the disadvantage of being time-consuming, but gave consistently good recoveries and was the method of choice where actual recovery of dextran sulphate or an accurate estimate of concentration was required.

When it was necessary to obtain an approximate estimation of dextran sulphate concentration in the presence of protein, and where actual recovery of material was not of importance, the modified one-stage prothrombin technique was useful. Results were not so precise as those obtained with the phenol method. Nevertheless, this method was found to be useful for routine laboratory use where many approximate estimates of dextran sulphate were required in a short period.

The "prothromboplastin effect" of dextran sulphate in low concentrations noted here has also been reported by Forwell and Ingram (1956). They considered that it was due to an acceleration of the interaction between Factors V and VII and brain thromboplastin. This aspect has also recently been discussed by Hjört and Stormorken (1957).

SUMMARY

Estimations of dextran sulphates in "protein-free" solution were made accurately by MacIntosh's colorimetric method which depends upon the production of metachromasia with certain basic dyes. Traces of plasma proteins or isolated serum albumin were found to invalidate this technique. Attempts to remove plasma proteins by preliminary protein precipitation or enzyme digestion were not satisfactory. Precipitation with phenol was found to remove protein most efficiently and allowed the dextran sulphate to be recovered almost completely. Two methods for estimating dextran sulphate, whilst still in the presence of protein, were tested: the first, based upon measurement of the anti-complementary activity, was unsatisfactory; the second, based upon measurement of the anti-thromboplastin activity, proved useful where a rapid, but approximate, answer was required.

Our thanks are due to Dr. C. R. Ricketts, who supplied the dextran sulphates, Mr. M. Hall for analytical data on the heparin, and Professor J. R. Squire for helpful criticism. One of us (H. A. E.) was in receipt of a research grant from the United Birmingham Hospitals Endowment Fund.

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GASTRIC PROTEOLYSIS IN DISEASE

3. THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE IN CHRONIC HYPOCHROMIC ANAEMIA AND IN IDIOPATHIC STEATORRHOEA

BY

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In chronic hypochromic anaemia the mean pepsin concentration of the gastric juice is reduced and in some patients the enzyme is totally absent (Davies, 1931; Castle, Heath, and Strauss, 1931; Meulengracht, 1932; Hartfall and Witts, 1933; Griffiths, 1934; Mullins and Flood, 1935; Aitken, Spray, and Walters, 1954). Aitken *et al.* (1954) divide their patients into those with and without achlorhydria. Complete absence of pepsin was found only among the former and the mean pepsin concentration in this group was less than a quarter of that in the patients who secreted free acid.

In idiopathic steatorrhoea the pepsin concentration of the gastric juice has been determined by Polland and Bloomfield (1930) in one case, Castle *et al.* (1931) in one case, and Aitken *et al.* (1954) in 10 cases. In six of these cases the gastric juice had a normal pepsin concentration, in four a subnormal concentration, and in two it was pepsin-free.

Slight proteolytic activity of gastric juice has also been detected at neutral *p*H in chronic hypochromic anaemia (Griffiths, 1934; Lasch, 1937; Jones and Wilkinson, 1938), and in idiopathic steatorrhoea in remission but not in relapse (Fox, 1949).

In both diseases, therefore, a reduced secretion of pepsin confirms the evidence for gastric dysfunction which is derived from gastric mucosal biopsy and from the reduced secretion of gastric hydrochloric acid. There is, however, an underlying assumption that diminished proteolytic activity results from a simple quantitative deficiency of normal pepsin. Estimations of gastric proteolytic activity have been carried out only near *p*H 2 and 7. The possibility that the gastric secretion in these diseases may contain enzymes different from normal, or differing proportions of normal enzymes, as is the case in pernicious anaemia (Taylor, 1959d), does not seem to have been considered. An investigation of this possibility is now described.

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Materials and Methods

The methods and materials used have been described previously (Taylor, 1956, 1957, 1959a).

Results

Chronic Hypochromic Anaemia.—Curves of *p*H activity for digestion of plasma protein by gastric juice have been determined in 10 patients (Table I and Fig. 1). Of these, one secreted juice without activity below *p*H 5. Two gave essentially normal curves with two maxima occurring in the *p*H ranges 1.8 to 2.4 and 3.5 to 3.7. One patient gave a curve with three maxima, at *p*H 1.5, 2.9, and 3.9, which resembled curves shown by a minority of normal subjects (Taylor, 1959a). Of

TABLE I

*p*H MAXIMA FOR DIGESTION OF PROTEINS BELOW *p*H 5 BY GASTRIC JUICE OF PATIENTS WITH CHRONIC HYPOCHROMIC ANAEMIA

Subject and Substrate	*p*H 1·5 to 2·4		*p*H 2·5 to 3·2		*p*H 3·3 to 5·0	
	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)
Plasma protein						
1. Histamine juice* ..	Inactive		Inactive		Inactive	
2. ,, ,,† ..	2·4	0·53	—	—	3·5	0·48
3. ,, ,,† ..	1·8	0·54	—	—	3·7	0·51
4. ,, ,,* ..	1·5	0·65	2·9	0·49	3·9	0·50
5. ,, ,,* ..	1·8	0·32	3·0	0·35	Declining activity	
6. ,, ,, ..	1·8	0·67	3·2	0·75	,, ,,	
7. ,, ,, ..	1·7	0·64	3·1	0·70	,, ,,	
8. ,, ,, ..	2·0	0·92	3·1	0·90	,, ,,	
9. ,, ,, ..	2·2	0·56	3·2	0·50	,, ,,	
10. ,, ,, ..	Declining activity		2·6	1·25	3·6	1·14
5% Egg albumen						
2. Histamine juice†	1·6	0·42	Declining activity		Declining activity	
6. Resting juice	1·6	0·48	,, ,,		Inactive	

In the tables and figures the amount of liberated amino-acid N is that of the whole digest, which consisted of 5 ml. buffer, 1 or 2 ml. of gastric juice, and 1 ml. of plasma or 5% egg albumen.

* Patients with achlorhydria.

† Patients with chronic blood loss.

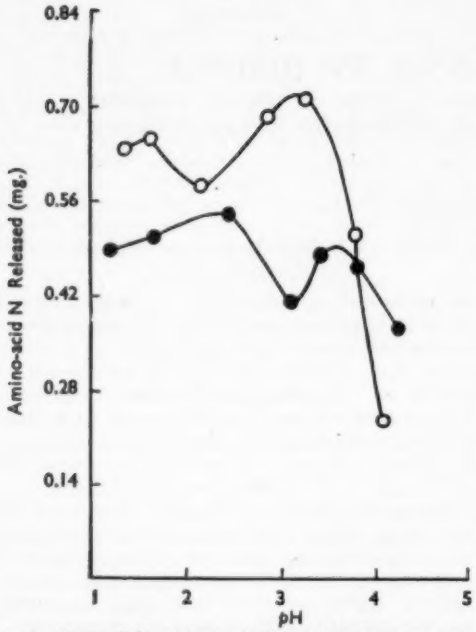

FIG. 1.—Curves of *p*H activity for the digestion of plasma protein by the gastric juice of two patients with chronic hypochromic anaemia. One (●) had suffered chronic blood loss and one (○) from idiopathic hypochromic anaemia. 37° C., 3 hr.

TABLE II

*p*H MAXIMA FOR DIGESTION OF PROTEINS AT *p*H 5 to 8·5 BY GASTRIC JUICE FROM PATIENTS WITH CHRONIC HYPOCHROMIC ANAEMIA AND IDIOPATHIC STEATORRHOEA

Subject	Substrate	*p*H Maximum	Amino-acid N (mg.)
Chronic hypochromic anaemia			
1	Plasma	7·6	0·12
2		6·9	0·14
3		Inactive	
5		"	
6		Active, no maximum	
7		" Inactive "	
9		" "	
Idiopathic steatorrhoea			
1	Plasma	6·8	0·08
2		7·2	0·10
6		7·2	0·13
5	5% serum albumin	Active, no maximum	

the remaining six patients, five gave curves with two maxima, which fell within the *p*H ranges 1.5 to 2.2 and 2.9 to 3.2. These ranges are those associated with the proteolytic activity of pyloric mucosal extracts in normal subjects (Taylor, 1959b). The remaining patient gave a *p*H activity curve with double maxima at *p*H 2.6 and 3.6. Such a finding has not been made in normal subjects.

Curves of *p*H activity for the digestion of egg albumen by gastric juice were determined in two subjects. Both showed only a single maximum at *p*H 1.6. One of these subjects had shown with plasma a normal digestion curve with two maxima and the other a curve of the "pyloric" type. These curves are similar to those found when normal gastric juice digests egg albumen.

Observations on the proteolytic activity of gastric juice upon plasma protein at *p*H 5 to 8.5 were made in seven patients of whom three exhibited no activity (Table II). Two showed slight activity at *p*H 7.4 to 7.7, but no maximum could be ascertained. Two gave curves with single maxima, one at *p*H 6.9 and the other at *p*H 7.6. These results do not differ from those obtained in normal subjects (Taylor, 1956, 1959c).

Idiopathic Steatorrhoea.—The gastric juice of four patients digested plasma protein below *p*H 5 with maxima in the *p*H ranges 1.5 to 1.9 and 2.8 to 3.0 (Table III, Fig. 2). These maxima are similar to those shown by human pyloric mucosal extracts. The gastric juice of one of these patients

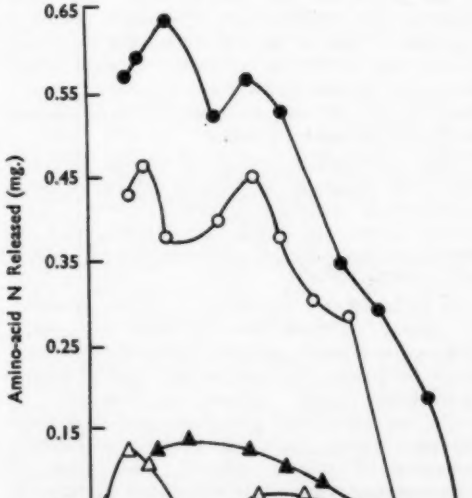

FIG. 2.—Curves of *p*H activity for the digestion of protein substrates by the gastric juice of four patients wih idiopathic steatorrhoea. Plasma protein (●○△), serum albumin (▲), 37° C., 3 hr.

TABLE III

*p*H MAXIMA FOR DIGESTION OF PROTEINS BELOW *p*H 5 BY GASTRIC JUICE OF PATIENTS WITH IDIOPATHIC STEATORRHOEA

Subject and Substrate	*p*H 1·5 to 2·4		*p*H 2·5 to 3·2		*p*H 3·3 to 4·0	
	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino acid N (mg.)
Plasma protein						
1. Histamine juice	1·9	0·62	2·8	0·56	Declining activity	
2. " "	1·9	0·64	3·0	0·59	"	"
3. " "*	1·5	0·12	3·0	0·07	"	"
4. " "*	1·5	0·46	2·8	0·45	"	"
5% *Human serum albumin*						
5. Histamine juice*	2·2	0·13	Declining activity		"	"
2% *Casein*						
4. Histamine juice*	1·8	0·14	2·6	0·12	"	"
5% *Egg albumen*						
4. Histamine juice*	1·6	0·14	Declining activity		"	"

* Patients with achlorhydria.

was also incubated with casein, giving a *p*H activity curve with maxima at 1.8 and 2.6, and with egg albumen, giving a single maximum at *p*H 1.6. In a fifth patient human serum albumin was used as substrate and only a single maximum at *p*H 2.2 was observed.

The juices, which were not bile stained, of three patients also digested plasma protein weakly at *p*H 6 to 8 with maxima at *p*H 6.8 to 7.2. In a fourth patient a very slight hydrolysis of human serum albumin at *p*H 7.1 could be demonstrated. These results are similar to those observed in normal subjects (Taylor, 1959c).

Discussion

Only the two patients whose anaemia resulted from chronic blood loss exhibited normal gastric proteolytic *p*H activity curves below *p*H 5, although one patient gave a curve with three maxima, which may perhaps also be considered normal. The principal difference from normal in the other patients was the occurrence of pyloric-type curves. A group of similar pyloric-type curves was found in patients with pernicious anaemia (Taylor, 1959d), a disease in which it is known that the mucosa of the body and fundus is atrophied whereas that of the pylorus may be preserved. Gross atrophy of the fundus and body of the stomach, as severe as that seen in pernicious anaemia, has also been observed in hypochromic anaemia but is relatively rare, occurring only in about 10% of patients (Lees and Rosenthal, 1958). Severe gastric mucosal atrophy might therefore perhaps account for the inactive juice of one patient but is unlikely to account for the pyloric pattern that has been observed in as many as five of 10 patients, especially as four of them could still secrete gastric acid.

A more likely explanation of the prevalence of the pyloric pattern in hypochromic anaemia is an alteration in the proportions of "pyloric" and "fundic" pepsins (Taylor, 1959b) secreted by the non-pyloric parts of the stomach. There is evidence that the neck chief cells of the fundic glands, which histologically resemble the cells of the pyloric glands, normally secrete a different pepsin from the "true" chief cells (Linderstrøm-Lang, Holter, and Ohlsen, 1935; Linderstrøm-Lang, 1952) and there is circumstantial evidence (Taylor, 1959b) that this enzyme of the neck chief cells resembles pyloric pepsin in activity. In normal gastric juice, however, fundic pepsin from the "true" chief cells preponderates. In hypochromic anaemia there is a tendency for the neck chief cells to proliferate at the expense of the "true" chief cells. Lees and Rosenthal (1958) found that in four of 19 patients there was "marked replacement of normal by pseudo-pyloric glands." Such a replacement of "true" chief cells by cells of the pyloric type might result in "pyloric" pepsin becoming the predominant enzyme, and it would thus be possible to explain the pyloric type of *p*H activity curve which has been observed.

In idiopathic steatorrhoea gastric mucosal atrophy varies from slight to very severe, and it is possible that a similar explanation to that advanced in hypochromic anaemia may account for the predominance of pyloric-type proteolytic *p*H activity curves.

Only in one patient with hypochromic anaemia (No. 10) and one with idiopathic steatorrhoea (No. 5) is it impossible to explain the proteolytic *p*H activity curves in terms of normally occurring gastric enzymes. In both these patients, and especially in No. 10, whose gastric juice was very active, the deviation from normal is unlikely to be erroneous, and the possibility that abnormal pepsins may have been elaborated arises. In general, however, it is evident that in hypochromic anaemia and idiopathic steatorrhoea the abnormal gastric proteolytic *p*H activity curves are adequately explained by postulating the secretion of abnormal proportions of normal enzymes so that these diseases stand in contrast to pernicious anaemia, in which some abnormal curves can only be explained by postulating the synthesis of abnormal and pathological pepsins.

Summary

The proteolytic activity of the gastric juice from 10 patients with chronic hypochromic anaemia and five patients with idiopathic steatorrhoea has been investigated at *p*H 1.5 to 8.

The pattern of activity at *p*H 5 to 8.5 did not differ from that of normal subjects.

Below *p*H 5 only three patients with hypochromic anaemia exhibited normal *p*H activity curves. One secreted inactive gastric juice. Five patients with hypochromic anaemia and four with idiopathic steatorrhoea exhibited *p*H activity curves resembling those of normal pyloric mucosal extracts.

It is suggested that in both diseases these pyloric-type curves result from the secretion of pyloric-type pepsin from the gastric mucosa in relatively increased amounts.

I wish to thank Miss Barbara Hunt and Mr. Brian Collett for skilful assistance with part of this work, the patients who donated gastric juice, many members of the Nuffield Department of Clinical Medicine for collecting gastric juice, and Mr. J. R. P. O'Brien and Dr. R. B. Fisher for reading the D.M. thesis from which this paper is abridged.

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J. clin. Path. (1959), **12**, 477.

THE PRESERVATION OF HUMAN BONE MARROW AT −79° C.

A TEMPERATURE-CONTROLLED METHOD OF TWO-STAGE COOLING

BY

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Lorenz, Uphoff, Reid, and Shelton (1951) showed that mice which had received lethal doses of ionizing radiation could recover if injected intravenously with viable bone marrow cells. Barnes and Loutit (1955) showed that erythropoietic cells of the infant mouse spleen, frozen by the technique of Polge and Lovelock (1952) and stored at −79° C. for periods up to 83 days, would also protect mice against lethal doses of *x* radiation. Ferrebee, Billen, Urso, Wan Ching Lu, Thomas, and Congdon (1957) demonstrated that mouse bone marrow suspensions could be preserved in this manner, and in a series of careful experiments obtained maximum survival of irradiated mice by suspending the marrow cells in tissue culture fluid with 15% glycerol, and freezing them at not more than 1° C. per minute to −15° C. and thereafter at not more than 10° C. per minute. The suspensions were prepared for injection by rapid thawing followed by reduction of the glycerol concentration with hypertonic glucose (Lovelock, 1952; Sloviter, 1956). It had been shown by Barnes and his co-workers (Barnes, Corp, Loutit, and Neal, 1956) that murine leukaemia could be treated by total body radiation followed by bone marrow transfusions, and in 1957 Thomas, Lochte, Wan Ching Lu, and Ferrebee reported a human case of chronic lymphatic leukaemia treated with total body radiation to a tissue dose of 250 r. followed by infusion of isologous stored marrow prepared by Ferrebee's technique. Good evidence was presented that the grafted marrow functioned for at least 43 days.

It was clear that for clinical work in the human being a reliable apparatus was required to freeze and store large volumes of marrow cell suspension in this manner. It was considered desirable that the machine should be compact and portable, fully automatic and yet simple to construct and operate. A degree of flexibility would also be useful should further experiments show different cooling rates to be preferable.

The Apparatus

Initially a replica of the apparatus of Polge and Lovelock (1952) based on a 1-litre polythene beaker was constructed, but, as Barnes and Loutit found, this failed to produce a satisfactory cooling curve (Fig. 1). It should, however, be noted that, whereas Polge pre-cooled his apparatus to between 2° and 5° C. before immersion in the −79° C. bath, we wished to start at room temperature. The addition of a heating coil to slow down the initial fall in temperature (Barnes and Loutit, 1955) produced a much improved gradient, but for clinical use the apparatus had several disadvantages. In the first

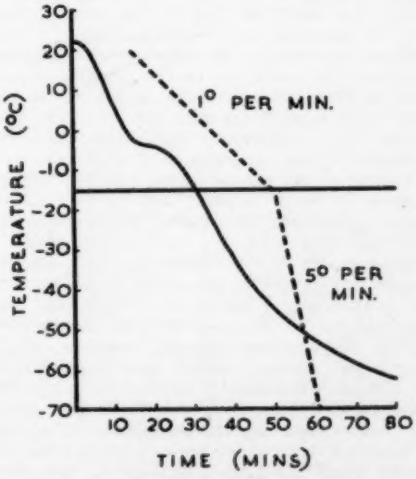


FIG. 1.—Cooling curve of Polge apparatus.

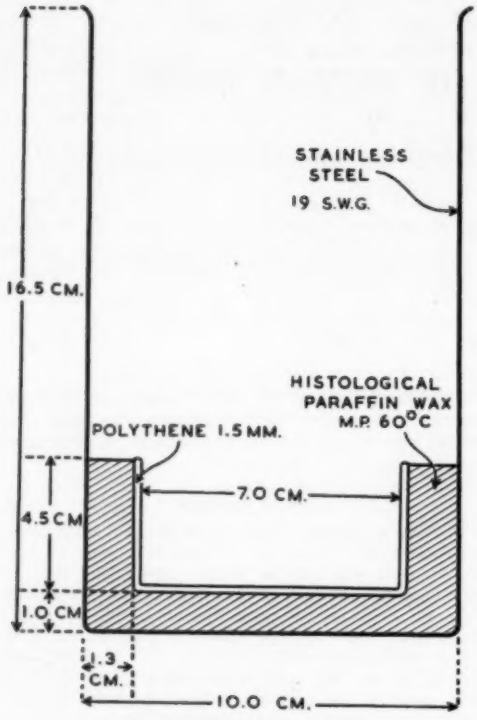


FIG. 2.—Dimensions of cooling vessel.

place the temperature in the inner beaker was not uniform; this was rectified by fitting a thick insulating lid of tufnol and incorporating a small stirrer to circulate the spirit thoroughly. More serious was the fact that the thermocouple measuring the temperature inside one of the ampoules had to be read at 30-second intervals to estimate the heating current required, while the inertia of the system made a smooth fall in temperature very difficult to obtain. The failure of the apparatus to produce cooling in excess of 2° C. per minute below −15° C. was also considered to be a serious fault in view of the evidence presented by Smith (1954) that the temperature zone between −15° C. and −25° C. should be passed in two minutes or less. Thus the minimum cooling rate of the second stage had to be 5° C. per minute. Experiments showed that rapid cooling below −15° C. could be obtained only if the insulation of the cooling vessel was radically reduced, although this naturally made slow initial cooling more difficult. However, a satisfactory compromise was provided by insulating the lower part of the beaker only; partial immersion in the −79° C. bath then produced slow cooling while complete immersion produced much more rapid cooling. It was found that suitable insulation could be provided by a layer of histological embedding wax (m.p. 60° C.) about 1 cm. thick placed inside the lower third of a 1-litre stainless steel beaker and held in place by a thin (1.5 mm.) polythene container. The precise dimensions are shown in Fig. 2. It was found that a very close approximation to the desired two-stage curve could be produced if the beaker was first immersed to a depth of 5 cm. and then, when the contents had reached −15° C., lowered to a depth of 12 cm. (Fig. 3). In order to obtain reproducible results it was essential to standardize the temperature of the vessel before cooling started (22° C.), the volume of methylated spirit it contained (520 ml.), and the size, content, and number of ampoules (19 10-ml. ampoules each containing 7 ml. of cell suspension in 15% glycerol). When less than 19 ampoules were being handled the total was made up with dummy ampoules containing 15% glycerol.

The temperature in the beaker is recorded automatically by a platinum resistance thermometer incorporating a 3 in. (7.7 cm.) pen recorder (Record Electrical Ltd.). The platinum resistance used has a fundamental interval of 180 ohms, but it is probable that a standard commercial model with suitable amplification would be satisfactory. A simple D.C. Wheatstone network has proved quite adequate. The amplifier is a three-stage balanced D.C. transistor circuit feeding a 1 m/a f.s.d. meter. The recorder employs a chart speed of 4 in. (10.2 cm.) per hour and is calibrated to read from +30° C. to −70° C. (Fig. 4).

Automatic lowering of the beaker from a depth of 5 cm. to the 12 cm. level is achieved by a thermostatically operated circuit (Fig. 5). The beaker is suspended from one end of a counterpoised arm. This is balanced so that the beaker will drop slowly when the solenoid which supports the arm is

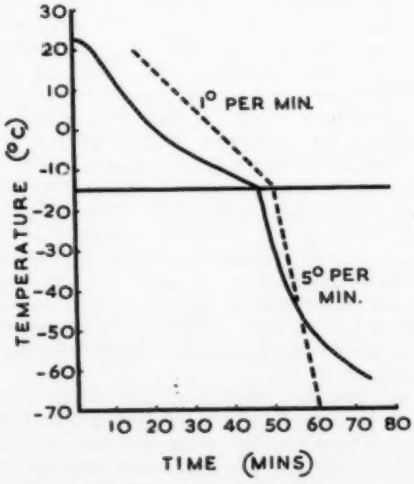


FIG. 3.—Cooling curve of modified apparatus.

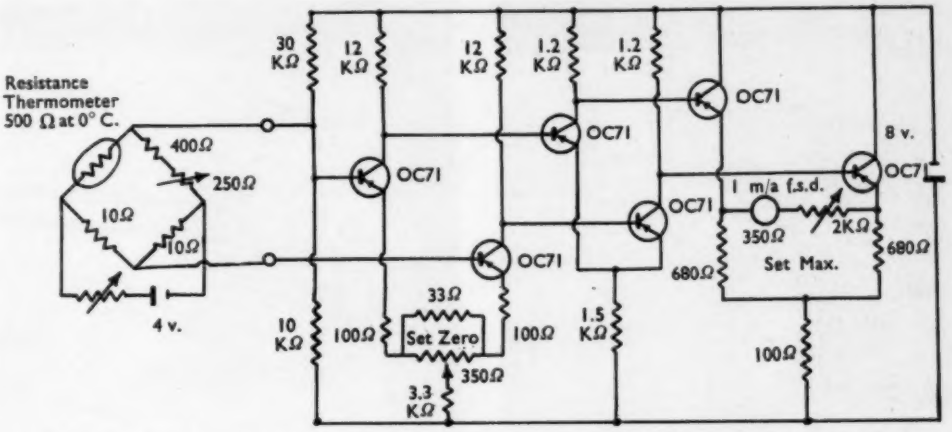
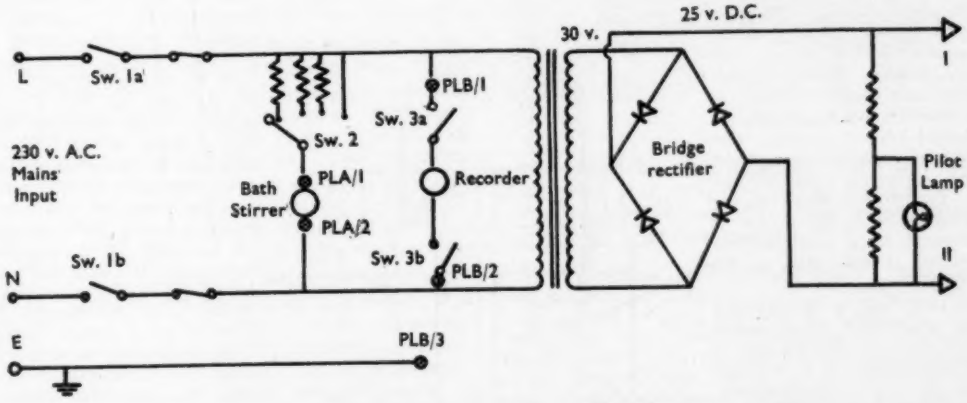


FIG. 4.—Circuit diagram of recording thermometer. The O.C.71 transistors are mounted in a large brass heat sink to minimize thermal effects.



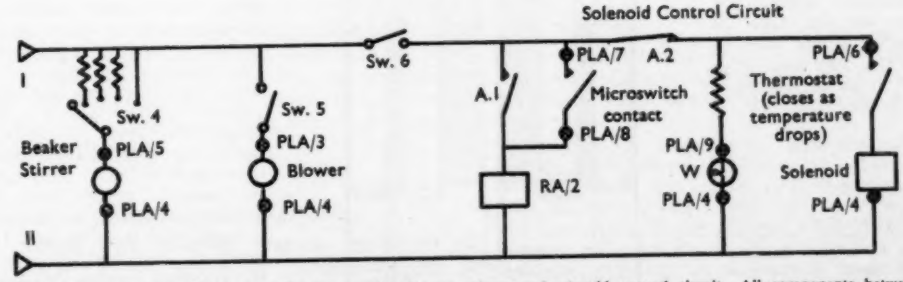
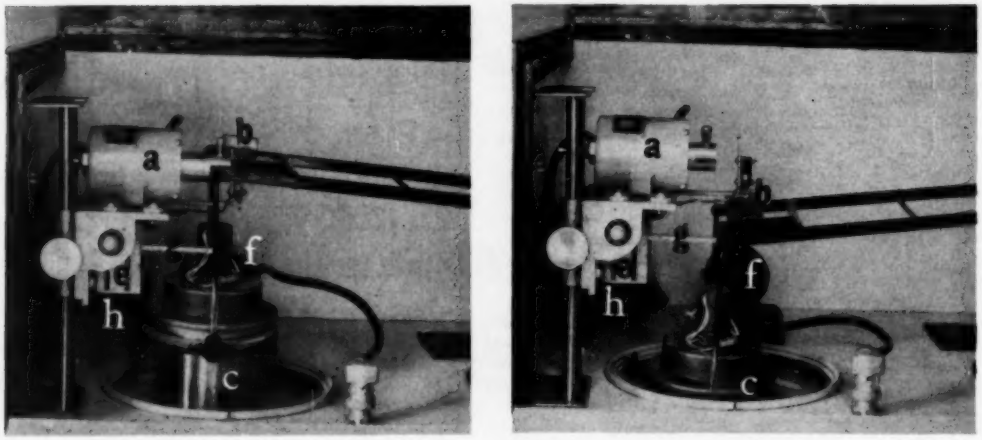


FIG. 5.—Circuit diagram of power supplies for recorder, blower, stirrers and solenoid control circuit. All components between points X—X are mounted remotely from the chassis. PLA: 12-pin outlet socket; PLB: 3-pin outlet socket for thermometer chassis only. W: warning light mounted on solenoid to indicate that microswitch has operated. To reset, Sw. 6 must be switched off and on again.



A B

FIG. 6.—Modified apparatus (A) cooling at 1° C. per minute and (B) cooling at 5° C. per minute. (a) Solenoid (b) Microswitch assembly (c) Cooling vessel (d) Dewar flask of "cardice" and methylated spirit (e) Adjustable clamp for stop (g) (f) Stirrer (g) Stop (h) Blower

energized. The thermostat controlling the solenoid is set to close at −15° C. and is immersed in the beaker with the ampoules. As the counterpoised arm drops it actuates a microswitch which breaks the solenoid control circuit. The arm is arrested by a stop placed 7 cm. below the solenoid (Fig. 6). Excessive frothing during the lowering of the beaker is prevented by a small blower mounted at the edge of the −79° C. bath.

A rack holding 19 ampoules was constructed to fit inside the beaker and rest on top of the wax lining. It is arranged that the stirrer, thermometer, and thermostat, which are attached to the lid, take up their correct positions without disturbing the ampoules (Fig. 7). Fig. 8 shows the complete apparatus in use.

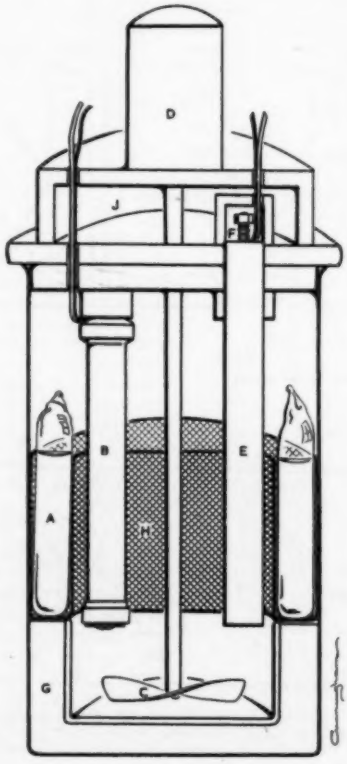


FIG. 7.—Cut-away view of cooling vessel. A, Ampoule. B, Platinum resistance thermometer. C, Stirrer. D, Stirrer motor. E, Thermostat. F, Thermostat setting screw. G, Insulating jacket. H, Ampoule rack. J, Tufnol cap.

It was found that when the temperature of an ampoule had fallen to −63° C. it could be immersed directly in spirit at −79° C. without exceeding the cooling rate of 10° C. per minute. It has therefore been our practice to transfer the ampoules to the storage flasks when they have reached −63° C. The ampoules are stored in quart Dewar flasks containing industrial spirit and solid carbon dioxide. The flasks are placed in a large insulated cabinet and surrounded by pieces of solid carbon dioxide.

The apparatus has been used to set up a bone marrow bank using ribs removed at thoracotomy for benign conditions. Schwartz and Tocantins' (1958) method is used to extract the marrow cells. The ribs are cut into 1 cm. fragments and each piece is crushed before being placed in a 250-ml. conical flask containing 30 ml. of heparinized tissue culture fluid. The flask is agitated gently for 20 minutes. The cell suspension is then freed from fat and filtered as described elsewhere for aspirated marrow (Newton, Humble, Wilson, Pegg, and Skinner, 1959).

The stored marrow has been administered with some success to two patients suffering from chronic lymphatic leukaemia treated by half-body irradiation, and to one patient who received large doses of HN_2 in connexion with surgery for carcinoma of the stomach. This had produced marked leucopenia and marrow hypoplasia, but following the marrow infusion the white cell count rose rapidly (Humble, Newton, and Kemp, 1958). Isologous stored marrow has also been administered to one patient suffering from aplastic anaemia due to chloramphenicol: no radiation or chemotherapy was given in this case and there was no evidence that the infused marrow survived.

Autologous infusion of stored marrow has also been practised in patients receiving wide-field, high-dose radiotherapy (Newton *et al.*, 1959) and in another series of patients who had received intensive chemotherapy for widespread malignant disease (Westbury, Humble, Newton, Skinner, and Pegg, 1959). It is noteworthy that stored marrow has been administered both intravenously and into the abdominal aorta without any evidence of harmful effect (Humble and Newton, 1958).

It would appear that treatment by marrow cell infusion should be considered in the type of case referred to above. It is also the logical treatment for accidental exposure to intense radiation, and in this connexion it is well to be prepared with large stocks of stored marrow. The possibility that human leukaemia may be successfully treated by total body radiation and marrow replacement remains and is being intensively studied here and elsewhere (Thomas *et al.*, 1957; Meighan and Bean, 1958; Reinhard, Brittingham, Moore, Holtz, Chaplin, Harrington, Loeb, Lessner, and Banson, 1958; Tocantins, 1958; Thomas, Lochte, and Ferrebee, 1959; Atkinson, Mahoney, Schwartz, and Hesch, 1959). Other interesting applications will undoubtedly present themselves as experience is gained with the method. Among these may be mentioned the possibility of organ grafting after irradiation and marrow replacement (Ferrebee and Merrill, 1957) and the modification of the severe homozygous states in the inherited haemolytic anaemias (Russell, Smith, and Lawson, 1956; Dameshek, 1957).

Summary and Conclusions

Viable bone marrow may be preserved at $-79°$ C. in 15% glycerol. The cooling rate is restricted to 1° C. per minute to $-15°$ C., after which a minimum of 5° C. per minute is required.

An apparatus is described whereby cell suspensions may be cooled in this manner.

Possible clinical uses for stored human bone marrow are briefly discussed.

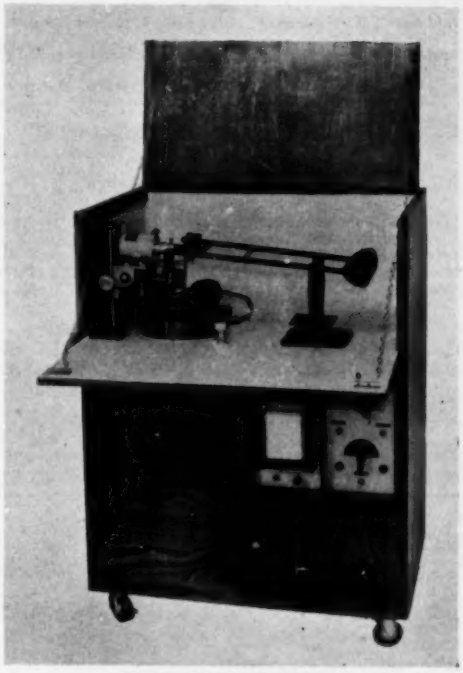


FIG. 8.—Complete apparatus.

We gratefully acknowledge the continued encouragement of Professor R. J. V. Pulvertaft, Dr. J. G. Humble, and Dr. N. H. Kemp, of the Department of Clinical Pathology, Westminster Medical School; also the enthusiastic collaboration of Sir Stanford Cade, Dr. K. A. Newton, and the staff of the Radiotherapy Department, Westminster Hospital.

The apparatus was constructed in the Physics Department under the direction of Mr. N. H. Pierce. We are especially grateful to Dr. C. W. Wilson, Chief Physicist to Westminster Hospital, for his interest in this work.

The platinum resistance was most kindly provided by Mr. J. W. Grant, of Elliot Bros. Ltd. (Messrs. Mullard kindly lent us the D.C. amplifier circuit). The illustrations were prepared by Dr. P. Hansell, of the Department of Photography, Westminster Medical School. The expense of construction of the apparatus was met by a generous grant from the Research Committee of the Board of Governors of Westminster Hospital.

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J. clin. Path. (1959), 12, 483.

TECHNICAL METHODS

A Standard for the Determination of Total Protein and Globulin in Biological Fluids

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(RECEIVED FOR PUBLICATION NOVEMBER 19, 1958)

Determination of Total Protein

Quantitative estimations of total protein in biological fluids may be performed by the micro Kjeldahl reaction with or without distillation (Rappaport and Lasowski, 1943), by a colorimetric method such as the biuret (Weichselbaum, 1946; Kingsley, 1940), by measuring the specific gravity according to the method of Phillips, Van Slyke, Emerson, Hamilton, and Archibald (1944), by the turbidimetric method (King and Wootton, 1956; Bossak, Rosenberg, and Harris, 1949), or according to the Klett manual.

Yeoman (1955) compared the iodometric Kjeldahl and turbidimetric methods using sulphosalicylic acid as reagent and found no significant differences between them, but for small laboratories it is important to use a simple turbidimetric method which is easy to perform. It must be remembered, however, that the turbidimetric methods are dependent on suitable standards.

Rappaport and Loew (1957) used in their colorimetric method a peptone solution as a standard for determining proteins in blood plasma.

A different standard suitable for turbidimetric determination is proposed for use in quantitative determinations of protein in cerebrospinal fluid, urine, etc.

In a similar fashion to Hiss (1904), who used water-diluted serum as nutrient medium for bacteria, it has been found that human sera diluted 1:100 in distilled water may be repeatedly autoclaved without coagulation and turbidity and may thus be used as standards.

The quantity of protein in a given sample of serum is determined before dilution either by the Kjeldahl method (Benhold, Kylin, and St. Ruszynyák, 1938) or by comparison with a peptone standard. Then this serum of now known protein content is diluted 1:100, autoclaved, and, if clear, distributed into vials; the vials are sealed and autoclaved again and may be kept indefinitely as standards. Sera containing abnormal globulin are not suitable for this purpose.

Principle of the Method

One part of cerebrospinal fluid and 6 parts of sulphosalicylic acid are mixed and the turbidity is measured in the improved Klett Summerson colorimeter using a sterile water-diluted serum for standard.

Reagents

(1) **Sulphosalicylic Acid.**—In a 3% aqueous solution, it keeps indefinitely.

(2) **Standard.**—One millilitre of human non-haemolytic serum of known protein content in 100 ml. distilled water, sterilized as described above (30 minutes at 115° C.), is distributed in 2 ml. quantities in vials, sealed, and sterilized again.

Procedure

(1) **Test Proper.**—To 3 ml. of 3% sulphosalicylic acid add 0.5 ml. of cerebrospinal fluid (or if only a small quantity of cerebrospinal fluid is available 1.8 ml. sulphosalicylic acid + 0.3 ml. cerebrospinal fluid).

(2) **Standard.**—To a second tube with 3 ml. of sulphosalicylic acid add 0.5 ml. of standard.

(3) **Blank.**—Three millilitres sulphosalicylic acid +0.5 ml. saline.

The protein concentration is calculated from the optical density read on filter (blue) 420 mn. using contents of tube 3 as blank and of tube 2 as standard.

The improved Klett colorimeter was used for readings (Rappaport and Eichorn, 1955).

The results of the total protein in the undiluted serum according to the colorimetric method of Weichselbaum (peptone standard) and diluted serum by the turbidimetric method (using Hiss water serum as standard) were identical during examination of a series of 40 sera.

Determination of Globulin in Cerebrospinal Fluid

The electrophoresis of cerebrospinal fluid is gaining importance in routine diagnostic work, but large quantities are needed and are not always available, therefore microanalysis, which gives the total protein and the globulin content, is important. In the literature many turbidimetric methods using ammonium-sulphate as reagent are described, and it is well known that the turbidimetric methods are the best when working with minute quantities of protein. The method of St. Ruszynyák (Yeoman, 1955) for the determination of globulin in cerebrospinal fluid was used here.

Principle of the Method.—Equal amounts of cerebrospinal fluid and neutral saturated ammonium sulphate are mixed. The turbidity is measured in the improved Klett-Summerson colorimeter using a sterile water-diluted serum as standard.

Reagents.—(1) Saturated solution of ammonium sulphate as used for the Nonne-Apelt reaction; the *p*H of the solution is corrected to *p*H 7, with sodium hydroxide or ammonium hydroxide.

(2) **Globulin Standard.**—The same as used in the determination of total protein in cerebrospinal fluid.

The globulin content of the standard solution in the vials is standardized either by a Kjeldahl or a biuret determination. When available Lederle (gamma) globulin diluted 1:200 can be used for this purpose as well. The diluted Lederle standard globulin coagulates on heating and so cannot be used as a sterilized standard.

An interesting phenomenon was observed while preparing the sterilized water-diluted serum. The total protein value determined in the diluted serum before and after sterilization remains constant, but the globulin value determined before and after sterilization showed a marked increase by this denaturation procedure. After renewed concentration as used for the electrophoresis of C.S.F. this solution showed only one globulin fraction and no albumin at all.

Procedure.—(1) Mix 1 ml. standard solution with 1 ml. of ammonium sulphate reagent.

(2) Mix 1 ml. of C.S.F. with 1 ml. of ammonium sulphate reagent.

(3) Mix 1 ml. of saline with 1 ml. ammonium sulphate reagent to be used as blank. Read in the colorimeter with a 420 mm. filter after five minutes.

The comparison of the results of globulin determinations in undiluted sera by the Weichselbaum colorimetric method and the turbidimetric one using diluted sera proved very satisfactory.

Summary

A method is described for the determination of total protein and globulin in biological fluids such as cerebrospinal fluid, urine, etc.

The standard is a sterile aqueous solution of serum which can be used for both the total protein and globulin determinations.

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Permanent Sickle Cell Preparations

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(RECEIVED FOR PUBLICATION FEBRUARY 6, 1959)

The usual methods for detecting sickling of red cells consist of the examination of wet preparations of red cells to which bacteria or a reducing agent are added. It would often be an advantage if stained preparations of sickle cells could be obtained for a "permanent" record, for teaching purposes, and for following the development of the sickling phenomenon in the newborn. Very few techniques have been described for the preparation of permanent films. Two methods are described below, the first simple and the second slightly more complex but even so still easy, rapid, and reliable with some advantages over the more simple technique. English (1958) recently described a simple method employing paraformaldehyde which had the disadvantage of liberating noxious formaldehyde fumes into the atmosphere. A technique without unpleasant fumes would be an advantage, and the first method described below is such a technique. Sherman (1940), while investigating techniques for the differentiation of sickle cell trait from sickle cell anaemia, took venous blood under anaerobic conditions and fixed the red cells immediately, taking care to exclude all oxygen until fixation was complete. This method did not reveal sickle cell trait. The basis of the second method, described below, is similar to the method of Sherman, except that the sickling desired is produced artificially by the addition of a reducing substance to a specimen of blood in an anticoagulant collected by venepuncture without any special precautions. Furthermore, using this method sickle cell trait can also be demonstrated.

Methods

Wet Preparations.—Two methods of making wet preparations to demonstrate the presence or absence of sickling were investigated: (1) using sodium diothionate (Itano and Pauling, 1949); (2) using 2% sodium metabisulphite (Lehmann, 1958).

The second technique proved most reliable and consistent, and was adopted for subsequent use.

Method 1.—Red cells were made to sickle in the usual way by mixing one drop of fresh 2% sodium metabisulphite on the centre of a 3 × 1 in. microscope slide with one drop of blood. A $\frac{3}{4}$ in. × $\frac{3}{4}$ in. coverslip was placed over the preparation and the edges were sealed round with paraffin wax/vaseline mixture. The slide was then allowed to stand on the

FIG. 1.—Wet preparation; 2% sodium metabisulphite; time taken for cells to sickle 90 min.

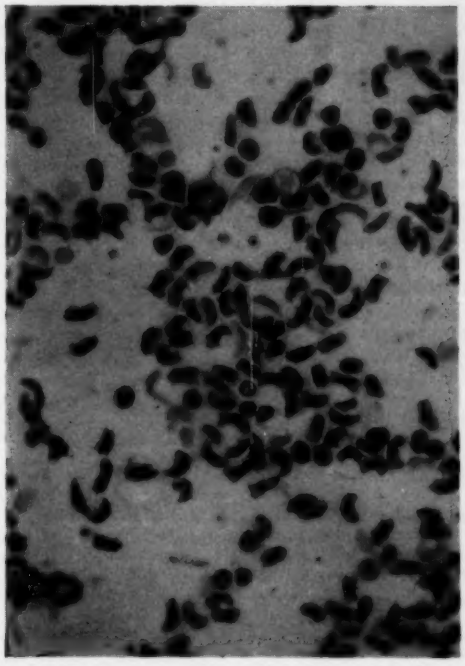

FIG. 2.—Dry stained preparation; tube method; time taken for cells to sickle 90 min.

bench at room temperature, being periodically examined microscopically for the presence of sickling. When sickling was considered to be complete, the coverslip was gently removed in a jet of hydrogen from a cylinder and held in the jet until the film was completely dry. The slide was then placed immediately in a Coplin jar containing acetone-free methyl alcohol and fixed for two minutes. The preparation was then stained in 0.5% alcoholic eosin for two minutes, washed in tap-water, dehydrated through graded alcohols, cleared in xylene, and mounted in "depex."

Method 2.—Into a round-bottomed 10 × 75 mm. tube was placed 0.2 ml. of blood that had been taken into a liquid anticoagulant and 0.4 ml. of fresh 2% sodium metabisulphite, and the suspension mixed. Liquid paraffin was then layered on the surface to a depth of ¼ in. This procedure was repeated in 10 tubes which were then allowed to stand on the bench at room temperature. After one hour had elapsed 2 ml. of 10% formal saline was introduced through the layer of liquid paraffin into the red cell/sodium metabisulphite mixture by means of a Pasteur pipette, making quite sure that no air bubbles were allowed to enter. The cells were allowed to fix for 30 minutes. This process was repeated in one tube every half-hour until all the tubes of cells had been fixed. After a time the cells began to settle in the tubes and it was necessary to keep them in suspension every half-hour by gently rotating the tubes by hand until the cells were once again evenly suspended.

After fixation was complete, the layer of liquid paraffin and a portion of the supernatant were removed by suction. The remaining cells were examined microscopically for sickling. If sickling was present films were made of the red cells on grease-free slides in the usual manner. They were allowed to dry in air and were then stained as described in method 1.

It was found by using the quantities of blood and sodium metabisulphite as described above that the time taken for the cells to sickle was exactly the same as the time taken by the cells in wet preparations and varied in different patients. Fig. 1 shows a wet preparation of cells from a patient with sickle cell trait, and Fig. 2 the same specimen of blood treated as in method 2. The time taken for the cells to sickle in this patient was 90 minutes.

Discussion

Method 1.—Although it is the quicker of the two techniques, method 1 has two disadvantages. The first and most important is that no matter how much

care is exercised in removing the coverslip an uneven film is the inevitable result. The second disadvantage is that if 12 films for teaching purposes are needed then 12 wet preparations have to be set up.

Method 2.—As regards Method 2, however, once the time taken for the cells to sickle has been discovered by the wet preparation, one tube can be set up for the equivalent time, and, using the quantities stated, upwards of 24 films can be prepared, all evenly spread. It follows that if permanent preparations are desired to show the stages of sickling formation this second method is most suitable.

The method of choice for regular, reliable, and most presentable preparations is as follows:

(1) Set up wet preparation and at the same time set up one tube as described in method 2 above.

(2) When the cells in the wet preparation have sickled, add formalin to the tube.

(3) After fixation is complete prepare films and stain as described above.

Summary

Two simple techniques for the permanent preparation of stained sickle cells are described.

In practice it has been found that if the wet preparation and the tube are set up at the same time, the formalin can be added to the tube when sickling is observed in the wet preparation.

I wish to thank Dr. R. F. Jennison for his advice and encouragement in writing up this method and Mr. B. Figg for the photographs.

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A Trial of Tablet Substitutes for the Gregersen Test

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From St. Thomas's Hospital, London

(RECEIVED FOR PUBLICATION FEBRUARY 2, 1959)

The Gregersen test for faecal occult blood employs benzidine as its chromogenic agent. Benzidine has been implicated as a factor inducing bladder tumours in man (Case, Hosker, McDonald, and Pearson, 1954; Baker, 1953). Supplies of Gregersen's test powders have, therefore, been curtailed and a substitute test has become necessary (Ogilvie, 1957).

Scrutiny of the literature suggested orthotolidine as a substitute (Ruttan and Hardisty, 1912; Kiefer, 1934; Gettler and Kaye, 1943; Hoerr, Bliss, and Kauffman, 1949; Peranio and Bruger, 1951; Hepler, Wong, and Pihl, 1953; Wilcox, 1956; Morgan and Roantree, 1957; Smith, 1958). Orthotolidine, though chemically related to benzidine, has not as yet been shown to have similar carcinogenic properties.

The Gregersen test, as modified by Needham and Simpson (1952), has been used at St. Thomas's Hospital for several years and has also been widely tested and established as clinically valuable in detecting faecal occult blood (Ogilvie, 1927; Meulengracht and Jensen, 1929; Dahl-Iversen and Nissen, 1930; Linn, 1949; Needham and Simpson, 1952; Hughes, 1952; Mendeloff, 1953).

For these reasons the Gregersen test was selected as the standard and the tablet tests, "hematest" and "occultest" (both containing orthotolidine), were selected for comparison. "Hematest" tablets are marketed as suitable for detecting faecal occult blood (Peranio and Bruger, 1951; Wilcox, 1956; Morgan and Roantree, 1957), and "occultest" tablets are marketed as suitable for detecting occult blood in urine (Watson-Williams, 1955).

Experimental

Materials.—Stools from 24 in-patients on a normal ward diet receiving phenindione as an anticoagulant were tested for occult blood. The testing of serial stools from these patients provided an extensive range of Gregersen test results; indeed, in one patient there was a range of Gregersen test results from negative to very strongly positive tests on melaena stools.

Method.—Portions of stool about the size of a walnut were collected in wax cartons and were tested in batches within three days of collection. Thin smears were made from each stool on three pieces of 11 cm. Whatman No. 1 filter paper, one piece being used for each of the three methods. The test tablet

was placed in the centre of the faecal smear and flooded with about 2 drops of tap water, sufficient water being used so as to form a moist circle about 1½ in. in diameter. The time taken for the first appearance of a blue ring to form around the tablet was noted, this ring being best seen in the small islands of white paper showing through the thin faecal smear. Any colour appearing on the tablet itself or on the reverse side of the paper under the tablet was ignored. The blue ring around the "hematest" tablet was usually small and developed slowly, while the ring formed around the "occultest" tablet developed rapidly and was larger.

The Gregersen test was performed by pouring about 1 ml. of Gregersen's solution (prepared by suspending 200 mg. barium peroxide and 25 mg. benzidine in 5 ml. glacial acetic acid) over the faecal smear on the third piece of filter paper. The time taken for the appearance of a blue-green or blue colour was noted. The solution was used within five minutes of preparation and was discarded if it turned green or blue.

Time Standards

The time standards for the Gregersen test were those suggested by Needham and Simpson (1952) and Bannerman (1957) and are set out in Table I. The "hematest" time standard was that suggested by the manufacturer of the tablet, a positive test being the formation of a blue ring within 120 seconds. The time standards for "occultest" and the intermediate ones for "hematest" were obtained by comparing the readings obtained by the tablet tests with those of the Gregersen test, and are presented in Table I.

TABLE I

EQUIVALENT TIME VALUES FOR GREGERSEN TEST, HEMATEST, AND OCCULTEST

Results		Gregersen Test	Hematest	Occultest
Strong positive	++	Blue in 5 sec.	Blue ring in 60 sec.	Blue ring in 35 sec.
Positive ..	+	,, ,, 15 ,,	Blue ring in 100 sec.	Blue ring in 75 sec.
Weak positive	+−	,, ,, 30 ,,	Blue ring in 120 sec.	Blue ring in 95 sec.
Negative ..	−	No colour change in 30 sec.	No blue ring in 120 sec.	No blue ring in 95 sec.

The agreement between the readings when analysed according to the degrees of positivity as shown in Table I was less than that obtained when the readings were simply classified as positive or negative. These latter results are presented in Tables II, III, and IV.

TABLE II

RESULTS IN 208 PAIRED READINGS OF GREGERSEN TEST AND HEMATEST

Hematest	Gregersen Test	
	0–30 sec. Positive	Over 30 Sec. Negative
0–120 sec. positive	153	6
Over 120 sec. negative ..	35	14

In 80% of the paired readings both tests agree, while in 17% the Gregersen test was positive while the "hematest" was negative. In the remaining 3% of the readings the Gregersen test was negative and the "hematest" positive.

TABLE III

RESULTS IN 160 PAIRED READINGS OF GREGERSEN TEST AND "OCCULTEST"

Occultest	Gregersen Test	
	0–30 Sec. Positive	Over 30 Sec. Negative
0–95 sec. positive	133	7
Over 95 sec. negative ..	12	8

In 88% of the paired readings, both tests agree, while in 7.5% the Gregersen test is positive and the "occultest" negative. In the remaining 4.5% the Gregersen test is negative and the "occultest" positive.

TABLE IV

RESULTS IN 160 PAIRED READINGS OF OCCULTEST AND HEMATEST

Occultest	Hematest	
	0–120 sec. Positive	Over 120 sec. Negative
0–95 sec. positive	106	35
Over 95 sec. negative ..	7	12

In 74% of the paired readings both tests agree, while in 22% "occultest" is positive and "hematest" negative. In the remaining 4% "occultest" is negative and "hematest" is positive.

Discussion

The agreement between the three tests is of the order which might be expected when using crude tests on faeces, a biological material of variable composition. The results show a similar trend to those of Smith (1958). "Occultest" may be considered to be slightly superior to "hematest" when the following factors are taken into account. "Occultest" is quicker, it gives a clearer end-point, and the readings, based on the criteria of positivity adopted, are in closer agreement with the Gregersen test than those of "hematest."

Summary

The carcinogenic properties of benzidine contained in Gregersen test powders have necessitated a substitute test for faecal occult blood.

Two single tablet tests utilizing orthotolidine as the chromogenic agent have been investigated for this purpose.

Two hundred and eight stools from 24 patients on anticoagulation therapy were tested for occult blood using the Gregersen test and "hematest" tablets. In 160 instances "occultest" tablets were also used.

Tabulating the readings as simply positive or negative there was 88% agreement between the

Gregersen test and "occultest," 80% agreement between the Gregersen test and "hematest," and 74% agreement between "hematest" and "occultest." "Occultest" appears to be a better substitute for the Gregersen test than "hematest."

"Hematest" and "occultest" reagent tablets were kindly supplied by Ames Company (London) Ltd.

I am grateful to Dr. G. I. C. Ingram and others for their help and encouragement.

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BOOK REVIEWS

A Primer in Medical Technology. By Paul M. Kraemer. (Pp. viii + 338; 33 figures. 58s. 6d.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1958.

This book contains 20 introductory lectures for student technicians. Four are devoted to the kidney and urine analysis, eight to haematology, seven to blood chemistry, and one to the cerebrospinal fluid.

The physiology of the kidney and liver, the pathogenesis of the anaemias, and blood group serology are discussed in an elementary manner; and there is a praiseworthy attempt to explain the intricacies of fluid balance. The coverage, however, is nowhere sufficiently complete to provide an examination text for British students, and some of the procedures suggested would not be acceptable here. Thus, for venepuncture, "the syringes . . . need not be sterile since nothing is injected, only withdrawn" (p. 73).

Much of the information relating to normal and abnormal function would be valuable to any student, but it is conveyed in far too many words. The style is often that of the popular lecture.

The limited scope and high price result in a text not likely to find favour with British students.

A. J. McCALL.

Haematological Technique, 2nd ed. By E. M. Darmady and S. G. T. Davenport. (Pp. viii + 244; 23 figures. 24s.) London: J. and A. Churchill. 1958.

That a second edition has been called for is a measure of the usefulness and demand for this handbook. The authors have obviously taken to heart the criticisms levelled at the first edition; extra sections have been added, and the whole presentation has been greatly improved. However, it is still not quite clear to whom the book is finally addressed; laboratory technicians will find two-thirds of it much to their liking, but such chapters, for example, as those on blood grouping, do not sufficiently fill in the background for them and at the same time are not nearly comprehensive enough for medical students. As a third edition will undoubtedly be needed the authors might well address that, and subsequent editions, wholly to laboratory technicians and at that moment, too, they might look again at the illustrations. The book is well produced and is of a useful size to be propped up on the bench.

A. GORDON SIGNY.

J. clin. Path. (1959), **12**, 489.

NEUROCHEMISTRY AND NEUROCHEMISTS*

BY

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From the Department of Chemical Pathology, Institute of Neurology, the National Hospital, Queen Square, London

A presidential address to a learned society or the inaugural address of a professor is usually devoted to one of three possible themes. It can be concerned with political considerations, with a historical survey of some subject, or it can be devoted to a purely scientific problem.

We, in our Association, have had in the past two of these themes, the political and the scientific. I am suggesting combining a historical approach with a scientific theme in relation to neurochemistry, and hope to show how in this century progress has been made in this particular field and how there can now be offered applications of the methods, techniques, and theories of the pure neurochemists to clinical medicine and neurology. Therefore the object is rather to show how basic research has frequently been of value at a later date in an understanding of disease processes and subsequently in the treatment of disease. Occasionally this order has had to be reversed and this will also be illustrated.

During the first 50 years of this decade there were 59 medical and physiological Nobel prize winners, and of these some 10 were biochemists or used biochemical methods in the field of neurology. Hence one in every six of all these Nobel prize winners was interested in the field which is under discussion, and some of these workers will be mentioned.

The chemistry of the nervous system can be said to have begun with Thudichum. He was born in Büdingen in Germany in 1828, and died of a cerebral haemorrhage in London in the year 1901. He qualified medically in 1851 at the University of Giessen, but had many other interests, one of which was singing, especially the singing of Italian arias. He was attracted to Liebig, thereby making his entry into the field of chemistry. However, like so many others, he had to flee the country of his birth, and, coming to England, he married in 1854 Charlotte Dupré, a sister of a chemist at the Westminster Hospital Medical School. He discovered urochrome in 1864, and suggested the name "lutein" for a certain colouring matter in animal tissues, a name later changed to lipochrome. His work on the chemistry of the brain took place during the years 1865 to 1882, and many substances that we now know much more about, such as sphingomyelin, kerasin, and cerebronic acid, were described and named by him subsequent to their preparation and identification. He measured the specific gravity of brain and also commented on the presence of such substances as copper in the brain. His monumental treatise, *Physiological Chemistry of the Brain*, appeared in London in 1884. Later in life, after he had retired, he turned his attention to food and wine, about which he wrote in 1895 and 1896.

As a direct result of his work a chemical section of the Kaiser Wilhelm Institute was started in Munich in 1928 largely through the activity of Kraepelin, who had been the first to devise a useful classification of schizophrenia. The name of Thudichum is remembered in another way, for I have discovered that in Galesburg in the state of Illinois, U.S.A., a psychiatric research department has named its laboratory after him. It might be worth mentioning now that this Thudichum laboratory, under the direction of Dr. Harold E. Himwich, is the chief psychiatric biochemical research centre in that state, and had officially given to it in 1957 a year's income of some £6,000 more than it is reported our Government gave for psychiatric research for the whole of Great Britain.

Another milestone was reached in 1937 when Page, then in New York, but later in Cleveland, published the volume entitled *Chemistry of the Brain*. He had worked earlier in Germany and knew many of Germany's prominent biochemists.

The subject of neurochemistry has now expanded well beyond the recognition of these earlier workers, and any general review of the subject is quite obviously impracticable. I have, therefore, chosen only a few major topics to discuss.

*The Presidential Address delivered to the Association of Clinical Pathologists on October 2, 1959.

Oxidative and Carbohydrate Metabolism

The brain has been shown to have a high rate of oxygen utilization. In fact, about 25% of the total oxygen used by the body in basal conditions is utilized by the brain, as was shown relatively recently in 1948 by Kety and Schmidt.

The brain makes use of glucose almost exclusively in its metabolism in contrast to other organs, and by sampling the arterial and the jugular venous blood it has been shown that the respiratory quotient is for practical purposes unity. Naturally, alterations in degrees of consciousness produce some alterations in utilization, and Himwich has made extensive investigations along these lines, and his and other authors' work is discussed in his book entitled *Brain Metabolism and Cerebral Disorders.*

Oxygen uptake varies from one tissue to another, the order of activity increasing from peripheral nerve to white matter to cerebral cortex. In the same way the trigeminal nucleus has a relatively low activity.

The method by which glucose is metabolized in the brain is very similar to that in other body tissues, following the pathway glucose-6-phosphate, fructose-6-phosphate, and fructose-1 : 6-diphosphate to 2 molecules of triosephosphate, and finally to pyruvate. There is then a conversion of pyruvate to a 2 C compound which by a complex reaction can enter the citric acid cycle, which has a close connexion with the Krebs cycle.

These basic portions of chemical knowledge, which are related to all body structures, had been obtained by pure chemists such as Krebs, a Nobel prize winner in 1953. Hans Adolph Krebs was born in Germany at Hildesheim some 59 years ago. After studying medicine he went to Berlin and then returned to Freiberg in Professor Thannhauser's clinic. He left Germany and came to Cambridge from whence he went to Sheffield and then to Oxford. In Cambridge in 1933 he worked on the synthesis of glutamine from glutamic acid and ammonia, while it was from Sheffield in 1937 that he published his work on the citric acid cycle. The tricarboxylic acid cycle is now recognized as the major pathway for the oxidation of carbon compounds in the body generally. However, unlike other tissues, the cerebral metabolism of available carbohydrate is not dependent on any local action of insulin. The work of Krebs embodies earlier theories of such workers as Szent-Györgyi and Martius and Lopp. Szent-Györgyi won his Nobel prize in 1937. He was born in Budapest in 1893 and qualified in 1917. However, he began research work as a first-year medical student. After a chequered career he finally became Director for the Institute of Muscle Research at Woods Hole, Massachusetts, where he is at present working.

I must mention two other workers in this field of basic science ; Carl and Gerty Cori, both born in Prague in 1896, eventually emigrating to America. They married in 1920, but had collaborated in research as class mates earlier. Their work, for which they were joint Nobel prize winners in 1947, was in relationship to glycogen, an important compound in carbohydrate metabolism.

I will mention very briefly the application of some of these various studies in so far as they affect us as practising clinical pathologists.

John P. Peters was born in 1887 and died in 1955. He was 34 years at Yale, and during that time he applied the biochemistry of carbohydrates to clinical medicine mainly in the fields of disorders of metabolism and more especially to diabetes. He is a joint author of the very well-known book *Quantitative Clinical Chemistry.*

Another person interested in carbohydrate metabolism who must be mentioned is Sir Rudolph Peters, who qualified in medicine in 1915, and after the first world war went to Cambridge and worked with Gowland Hopkins. In 1923 Hopkins offered Peters the chair of biochemistry at Oxford, where the laboratory had been started 18 months earlier by Benjamin Moore, who had been the occupant of the chair of biochemistry at Liverpool, the first ever to be created in England. Here at Oxford at the age of 33, Peters continued his pioneer studies, and much of his earlier work was on oxidation and the SH compounds. This followed investigations by Warburg and by Meyerhof, two very eminent German chemists.

Much of the work of Peters was on thiamine, and few others were interested in this subject at that time. He is now investigating new problems of the monofluorocarbon compounds in relation to vitamin deficiency.

One of the pupils of Peters is Thompson, who, while at Oxford with Peters, together with Stocken, developed during the last war 2,3-dimercaptopropanol, now known as dimercaprol (B.A.L.). This compound, as is well known, acts as a remover from the body of many heavy metals, including lead and copper. Thompson and Peters were interested in pyruvate metabolism ; the pyruvate oxidation system contains highly reactive thiol (SH) groups. Arsenicals combine

with these groups and pyruvate metabolism is thereby impaired. Coenzyme A also contains one of these essential SH groups, and this is the site at which B.A.L. competes, thereby offsetting the action of poisonous substances such as the arsenicals. More recently, Thompson has applied these principles to the question of peripheral neuritis and polyneuritis in man, and he has shown that in a percentage of cases of polyneuritis the fasting blood pyruvate level may be raised, and further that there is an abnormal rise in blood pyruvate after a test dose of glucose. He has shown further that, in a proportion of the patients who show this abnormal pyruvate tolerance test, vitamin B_1 used therapeutically is able to effect some degree of cure of the patient and the blood levels of pyruvate may return to normal.

Cerebral Lipids

The brain contains some 12% to 15% by wet weight of lipid, or 50% to 60% by dry weight. This fatty material was found by Thudichum to consist largely of cholesterol, sphingomyelin, and cerebroside. Neutral fat is also present but in very small quantities. The early workers used whole human brain and separated the substances on a macro basis with characterization of the component constituents. Thierfelder, for instance, at the beginning of this century, isolated one of the cerebrosides (cerebron), and 50 years later Chatagnon in Paris, by fresh experimental methods, obtained a substance which gave crystalline appearances and infra-red spectroscopic findings which were similar to those of Thierfelder's original preparation, which was still in existence and with which it was compared.

It is perhaps correct to state that the largest amount of work done on these substances in the human brain has taken place in the last 10 years. Nevertheless, in the 1920s Klenk, Remkamp, Deuel, and others were separating and characterizing many of these compounds on a purely chemical basis. Considering very briefly one or two compounds, one can say that Gmelin in 1826 found that cholesterol in the brain had the same formula and configuration as cholesterol in gall-stones, but it was later found that the esterified form of cholesterol did not exist in any appreciable amount in normal brain tissue.

Sphingomyelin, so named by Thudichum, was later found to contain a fatty acid which is probably the C 24 lignoceric acid, but it may also be stearic or nervonic acid. Sphingomyelin is closely related to cerebroside as can be seen in Fig. 1, from which it can be observed that sphingosine is a component part of not only sphingomyelin and cerebroside but also of ganglioside and possibly of a substance called cephalin B.

Klenk and his co-workers around 1940 found a compound in the brain which they named substance X, later renamed ganglioside, and it was shown that it contained neuraminic acid, hexosamine—probably a galactosamine—and a hexose radicle. There is still a good deal of controversy about the exact composition of ganglioside, for slightly varying views from those of Klenk have been expressed by both Svennerholm and Bogoch in the last two years. Klenk is a pure chemist and has built up at Cologne a famous department full of enthusiastic workers who are, however, also interested in disease processes.

Thannhauser and his colleagues in recent years have made important contributions to the chemistry of sphingomyelin and of the plasmalogens, originally described by Feulgen in muscle. He has had much to do with work on various diseases, such as Gaucher's disease and xanthomatosis, while working in America.

The school in Canada led by Rossiter had investigated the brains of normal infants and adults, estimating the relative amounts of sphingomyelin, cerebroside, cholesterol, and lecithin found in the white and grey matter, and their results have been confirmed by Brante in Sweden and also by the group working with me, all within the past 10 years. Just recently, we have been able to add, in an even more extensive series, the results of similar estimations of the various lipids in brains from the foetus, the full-term infant, the young infant, and the child.

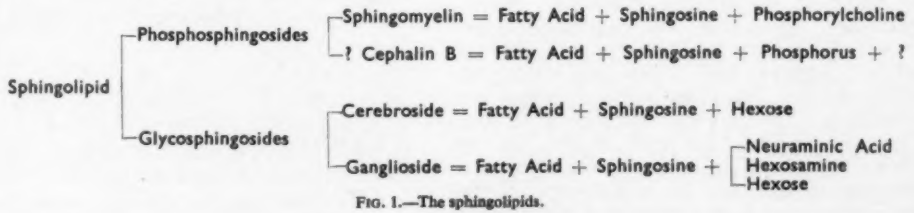
Sphingolipid
Phosphosphingosides
Sphingomyelin = Fatty Acid + Sphingosine + Phosphorylcholine
? Cephalin B = Fatty Acid + Sphingosine + Phosphorus + ?
Glycosphingosides
Cerebroside = Fatty Acid + Sphingosine + Hexose
Ganglioside = Fatty Acid + Sphingosine +
Neuraminic Acid
Hexosamine
Hexose

FIG. 1.—The sphingolipids.

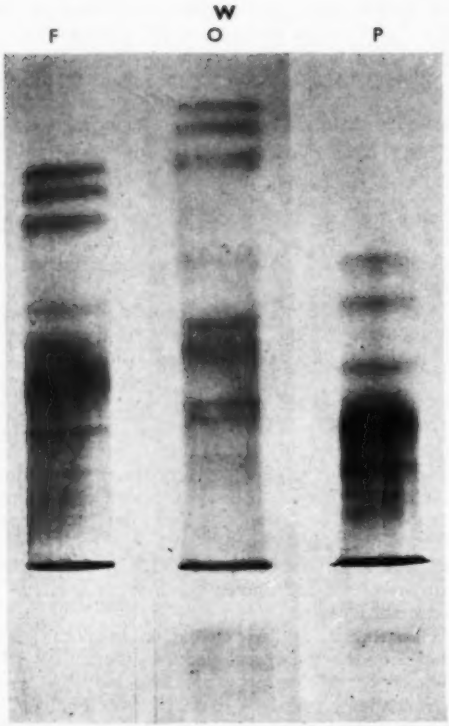

FIG. 2.—Starch electrophoresis of water-soluble proteins of normal cerebral white matter. F = frontal, O = occipital, P = parietal lobes.

In recent years cerebral metabolism has been investigated extensively. The modern trend is to consider everything from the dynamic aspect, and therefore investigators using radioactive isotopes have followed the metabolic processes of many of these substances. In the animal it has been shown by Waelsch and his colleagues that very little, if any, build-up of cholesterol takes place in the adult brain. This may also be true for phospholipids, but the position regarding these substances is not yet finalized.

The cerebral lipids do not, of course, constitute the whole of the cerebral substance. Water, electrolytes, amino-acids, and mucopolysaccharides are also present, but these must be supported and held together, and in part this is done by various protein substances, some of which are proteolipids. Total proteins constitute some 40% of the dry weight of the brain or 10% by wet weight. Knowledge of most of these proteins is extremely meagre, but the neurokeratin of the older writers may be the proteolipid which Folch and his colleagues have investigated as recently as 1951. Proteolipid is an important part of the myelin sheath, and Finean has represented the myelin sheath as being made up of alternate layers of lipid and of protein.

Recently a study has been carried out on the water-soluble proteins of the brain. Portions of cerebral white matter in 5 to 10 g. amounts were extracted with 10 to 20 ml. of 10% sucrose, and similar amounts of cerebral cortex extracted with 5 to 10 ml. quantities of sucrose. The extracts were concentrated, and the proteins present examined by starch electrophoresis. Different areas of the brain have been selected, and so far nine portions of the frontal lobe, seven from the parietal, and 10 from the occipital lobe have been examined with some slight differences in pattern being observed. The results are illustrated in Figs. 2 and 3. The patterns found do not appear to bear any relation to the proteins of the serum, which in some instances were put up in parallel with the brain extracts.

Interesting results, however, have been obtained in cases of suspected cerebral oedema. A well-

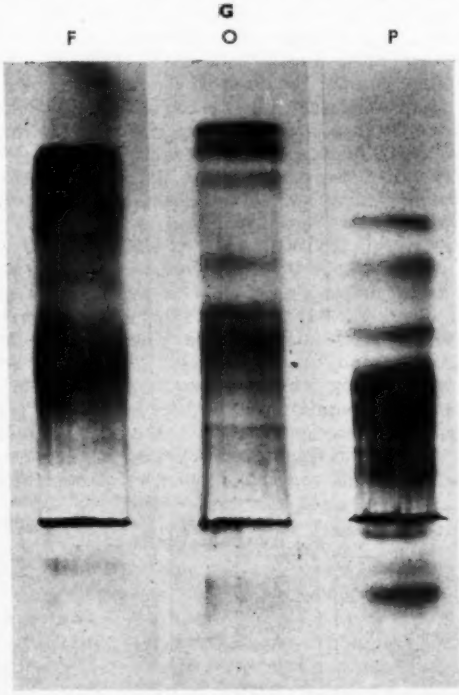

FIG. 3.—Starch electrophoresis of water-soluble proteins of normal cerebral cortex. F = frontal, O = occipital, P = parietal lobes.

marked band of protein has been demonstrated in the same region that albumin appears in a serum pattern in starch electrophoresis (Figs. 4 and 5). This albumin band has been found even in portions of brain which on naked-eye examination were not definitely oedematous. Macroscopic diagnosis of cerebral oedema is notoriously difficult as has been shown previously by histological examination as well as by chemical techniques, and this method appears to be a delicate one for its demonstration.

Much work has been done on the amino-acid composition of the various proteins, and Richter in England and Waelsch in America have worked extensively on this subject. Amino-acids are being metabolized continuously as has been shown by isotope studies, and some of them, such as glutamic acid, play a very important part in general metabolic processes, as was mentioned earlier when discussing the work of Krebs.

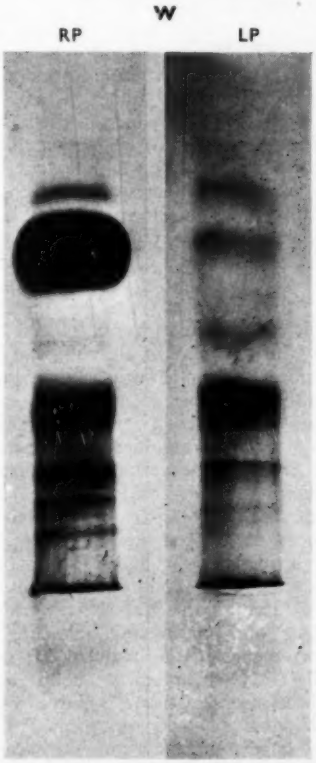

FIG. 4.—Oedematous right parietal and non-oedematous left parietal white matter. Starch electrophoresis.

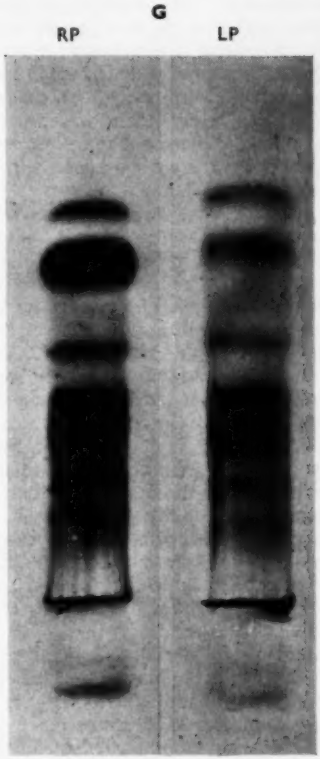

FIG. 5.—Oedematous right parietal and non-oedematous left parietal cortex. Starch electrophoresis.

Brain tissue contains both glutamic acid and its amide glutamine. There is no evidence to suggest that glutamic acid can replace glucose as a source of fuel. It can react with ammonia to form glutamine or be oxidized by glutamic dehydrogenase with the formation of ammonia. The later stages of such a cycle include α ketoglutaric acid, and this has a connexion with the Krebs cycle. It has been suggested that some of these pathways may be involved in hepatic coma.

Returning now to the lipids, the next links in the chain that should be considered are the experiments in 1948 on Wallerian degeneration by the group led by Rossiter. They cut the sciatic nerve in animals and at various stages of degeneration examined chemically the distal part of the nerve as well as control nerves. It was found that at first water increased in amount while total phospholipids decreased. Neutral fat decreased but later returned to normal levels.

The components of myelin—sphingomyelin, cerebroside, and free cholesterol—decreased, but esterified cholesterol from being absent in the intact and normal nerve increased appreciably. Proteins, probably proteolipid or neurokeratin, also decreased. Here then was experimental evidence relating to demyelination. Recent papers by this group have been concerned with various associated enzyme changes.

Some workers have applied these techniques to the various disease processes. It has, for instance, been shown in work which I did in 1953 that demyelination in multiple sclerosis followed a chemical pattern which in many ways was very similar to that found in the cut sciatic nerve. There is in multiple sclerosis in the active plaques, but not in chronic lesions, an increase of water and a loss of the myelin lipids, together with a well-marked increase in esterified cholesterol.

One of Klenk's pupils, Dr. Debuch, has shown that a substance, lysocephalin, possesses some properties similar to those of lysolecithin. It has been suggested that this might well produce demyelination locally within the myelin sheath, and this type of enzymic action has been proposed as a possible cause of demyelination in multiple sclerosis. Thompson and his colleagues are pursuing this aspect.

Very similar lipid chemical findings have been obtained in Schilder's disease or the so-called sudanophilic type of diffuse sclerosis. It has not yet been possible to demonstrate the factor that causes this demyelination, whether in fact it is enzymic in nature and whether it also has a genetic basis. The results that have been obtained in both multiple sclerosis and sudanophilic diffuse sclerosis are illustrated in Tables I and II, where it will be seen that there is a very great increase in the amount of esterified cholesterol, and this is absolutely characteristic of these two diseases. The deposition of esterified cholesterol in histological sections stained by Scharlach R stain red, as can be seen in Fig. 6.

Turning very briefly to the group of diseases known as the lipidoses, there is abundant evidence from the work of Thannhauser, Klenk, and my own group that some of the various substances which have been described by the chemists are increased in amount in these diseases. Thannhauser and his associates found in Niemann-Pick's disease affecting the peripheral organs that there was an increased content of sphingomyelin in these organs, and Klenk and I have both shown in patients in whom the nervous system is involved that there is an increased content of sphingomyelin and of cholesterol in the brain itself. We have also found an increased amount of ganglioside in the cerebral cortex.

TABLE I

CEREBRAL LIPIDS IN MULTIPLE SCLEROSIS

	Normal White	Demyelinated White
Total phospholipid ..	5·8	1·8
Sphingomyelin	2·4	0·5
Total cholesterol ..	4·1	2·8
Esterified ,, ..	0·3	1·7
Ganglioside		1·0
Water	69·0%	80·4%

Results in g./100 g. fresh tissue except ganglioside, which is in g./100 g. dry tissue.

TABLE II

CEREBRAL LIPIDS IN BIOPSY SPECIMENS OF SUDANOPHILIC DIFFUSE SCLEROSIS

	Cerebral White	Cerebral Cortex
Total phospholipid ..	4·1	3·35
Total cholesterol ..	2·4	1·21
Esterified ,, ..	0·8	0·17
Hexosamine	0·39	0·59
Ganglioside		1·5
Water	77·8%	82·6%

Results in g./100 g. fresh tissue except ganglioside and hexosamine, which are in g./100 g. dry tissue.

In amaurotic family idiocy Klenk and his associates in 1942 obtained raised levels of ganglioside in the brains of patients, and they found that the level of neuraminic acid, that is of ganglioside, showed an increase of even as much as five to 10 times the normal, and this increase occurred almost exclusively in the cerebral cortex where the ganglion cells can be seen by histology to be full of a P.A.S.-positive material.

During the past few years the application of these basic chemical facts has been used in clinical pathology in the field of neurology. Chemical investigations of the brains of patients dying from multiple sclerosis, from lipidoses, and from encephalitis have been made by me, and distinctive patterns obtained in these different diseases. This knowledge has been utilized in the examination of cerebral biopsy material. Numerous patients with progressive mental disturbances have been examined by clinicians without a definite diagnosis being possible, although some of the conditions are known to be familial. After all the facts have been given to the parents and permission obtained, a small sample of cerebral tissue, about 1 cm. cube, was removed. My histological colleagues have shared these pieces of tissue with me, and I have examined some 74 such specimens. A positive diagnosis has been made on 51 occasions by chemical examination, and in only one case have the histological and biochemical opinions been in

complete disagreement. It is not possible to give the complete details, but one or two examples will demonstrate the type of findings that have been obtained. (I must mention that no clinical defect has resulted, nor have we been able as yet to obtain post-mortem confirmation of our opinions, apart from three cases, and these patients died more than six months after operation.)

A patient with a diagnosis of amaurotic family idiocy arrived at histologically and chemically showed typical histological lesions in the cerebral cortex, and these can be seen in Figs. 7 and 8. The chemical examination revealed a marked increase of ganglioside in the absence of any evident loss of phospholipid or of demyelination (Table III).

TABLE III
CEREBRAL LIPIDS IN BIOPSY SPECIMENS FROM AMAUROTIC FAMILY IDIOCY

	Cerebral White	Cerebral Cortex	Normal Cortex
Total phospholipid ..	5·88	3·70	3·5–4·5
Total cholesterol ..	3·41	1·11	1·0–1·5
Esterified ,, ..	0	0	0
Hexosamine	0·36	0·70	0·5–0·7
Ganglioside		2·3	1·0–1·5
Water	75·1%	81·9%	82–85%

Results in g./100 g. fresh tissue except ganglioside and hexosamine, which are in g./100 g. dry tissue.

Metachromatic leucodystrophy, which is familial, shows an altered appearance of the myelin pattern, for in the white matter there is metachromasia as illustrated in Fig. 9, while the chemical findings are seen in Table IV. It is to be noted that there is a marked increase of hexosamine in the white matter, while there is also evidence of a moderate degree of demyelination.

TABLE IV
CEREBRAL LIPIDS IN BIOPSY SPECIMENS OF METACHROMATIC LEUCODYSTROPHY

	Cerebral White	Cerebral Cortex	Normal White
Total phospholipid ..	3·12	3·77	7·0–8·0
Total cholesterol ..	1·28	0·93	4·0–5·0
Esterified ,, ..	0·11	0·14	0
Hexosamine	0·48	0·74	0·2
Ganglioside		1·4	
Water	81·0%	83·3%	70–74%

Results in g./100 g. fresh tissue except ganglioside and hexosamine, which are in g./100 g. dry tissue.

It must be mentioned that metachromatic deposits occur in the kidney, and examination of the urine reveals such metachromatic material, and this was indeed found in this patient.

Lastly, subacute leucoencephalitis of the inclusion body or Van Bogaert type shows some slight but definite chemical findings, and are shown in Table V. There is only slight demyelination without the presence of cholesterol ester but with a slight increase in hexosamine levels.

TABLE V
CEREBRAL LIPIDS IN BIOPSY SPECIMENS OF SUBACUTE ENCEPHALITIS

	White	Cortex
Total phospholipid ..	5·4	4·0
Total cholesterol ..	2·7	1·2
Esterified ,, ..	0·05	0·02
Hexosamine	0·51	0·63
Ganglioside		1·0
Water	77·5%	81·1%

Results in g./100 g. fresh tissue except ganglioside and hexosamine, which are in g./100 g. dry tissue.

Table VI shows the actual number of cases which have been examined chemically, and the groups of diseases into which they fall.

TABLE VI
74 CEREBRAL BIOPSIES EXAMINED CHEMICALLY

Condition	Abnormality Diagnostic	Abnormal but Not Diagnostic	No Abnormality
Normal			26
Amaurotic family idiocy	10	5	
Metachromatic leucodystrophy	9		
Multiple sclerosis ..	1		
Sudanophilic diffuse sclerosis	4		
Subacute encephalitis ..		6	
Organic dementia ..		8	
Miscellaneous	1	4	

Acetylcholine

Hunt and Taveau in 1906 were the first to demonstrate that acetylation of choline (a substance known to Thudichum and obtained originally from phosphatide in bile, hence its name) resulted in a product of greatly increased pharmacological activity. Loewi demonstrated in 1921 that liberation of a substance now known as acetylcholine, but called by him "*Vagusstoff*," was present in the perfusing fluid of a frog's heart after stimulation of the vagus nerve. Sir Henry Dale in 1914, 1929, and 1933 very ably described the various effects of acetylcholine, and in the last paper of 1933 introduced the terms "cholinergic" and "adrenergic." Loewi and Dale shared the Nobel prize in 1936. In 1911 Dale, in association with Barger, was the first to isolate histamine from animal tissues. However, he is best known for his work on acetylcholine, which was later followed by work on neostigmine. Considerable knowledge is now available

concerning the synthesis and destruction of acetylcholine after its release at nerve endings and the conception of an acetylcholine cycle has been put forward. It was soon clear from experimental procedures that an enzyme must be present to convert acetylcholine into some less active form. Such an enzyme, cholinesterase, which has been found to be widely distributed in the body, can in fact break down acetylcholine to acetic acid and choline. Two main types of such an enzyme have been found and these have been called, in 1943, true and false cholinesterase, or specific or non-specific cholinesterase. Nachmansohn, who proposed the latter term in 1945, was also an able worker on acetylcholine.

These two cholinesterases have been found to act on different substrates, and are present in varying amounts in different organs and areas. True cholinesterase was found by Nachmansohn in 1939 to be located more in the cerebral cortex than in the cerebral white matter, while pseudo-cholinesterase was present to a greater extent in the white matter, as in fact was shown by Ord and Thompson in 1952.

After release of acetylcholine at the nerve endings and its hydrolysis some process must then be present for its resynthesis. Quastel and his co-workers in 1936 suggested such a process, and it has since been proved that acetyl-coenzyme A provides the acetyl group which is transferred to choline by the action of the enzyme choline acetylase. Acetyl-coenzyme A is probably derived from pyruvate, which links this mechanism, therefore, with the glucose cycle.

Only one or two applications of these findings to clinical neurology will be mentioned. Much biochemical work on epilepsy has been done at Montreal led by Elliott. Elliott and Tower in 1952 estimated bound acetylcholine and cholinesterase in normal portions of brain and in epileptogenic areas of brain, and found that in the latter there was a marked reduction of bound acetylcholine. Further work both by Elliott in Montreal and by Tower in Bethesda has followed, but it is too early to state its final practical value. Elliott has recently reported on γ amino-butyric acid which is present in brain tissue, and has said that both glutamate and γ amino-butyric acid may be "action substances" of neurophysiological importance, and that from his experimental results there may be a balance of effects of these two substances, and that they may determine neuronal activity.

The other condition I wish to mention is ginger paralysis. This is a form of motor neuritis first seen in the United States in 1930 following the drinking of a beverage containing an extract of Jamaica ginger. This was later found to contain tri-*ortho*-cresyl phosphate (T.O.C.P.). Various degenerative lesions were found in axis cylinders and in the myelin sheath in not only the peripheral neuritis mentioned but also after poisoning by T.O.C.P. present in other substances such as apiol and in certain cooking oils. The hen can be used as an experimental animal, and work has been done since 1950 on this subject by a number of experimenters. Earl and Thompson in 1952 showed that T.O.C.P. was a selective inhibitor of pseudocholinesterase in nervous tissue and in plasma but that true cholinesterase was unaffected. Certain new organophosphorus insecticides are also similarly dangerous, for parathion and mipafox both cause plasma and erythrocyte cholinesterases to be lowered. These last compounds do not, however, produce paralysis or degenerative lesions of nerve.

It is perhaps of interest to notice here that amongst some of these substances recently produced is one for locust control, and experimental work on this has just been published showing the effect of such substances on cholinesterases in the nerve cells of locusts.

So far an attempt has been made to show that our understanding of disease processes has followed a fairly definite pattern, and has stemmed from basic chemical work. Application of such basic knowledge has been applied to suitable patients with varying diseases in order to elucidate the cause and the nature of the pathological lesion involved. However, we now come to a group of disorders in which the reverse procedure has been followed. In patients with some diseases, abnormal chemical findings were first discovered, and, following this, basic chemical knowledge had to be determined. In 1908 Sir Archibald Garrod delivered the Croonian lectures at the Royal College of Physicians and his subject was "Inborn Errors of Metabolism." There are now known to be a number of such disorders, and one or two of them will be mentioned and an indication as to how progress has been made in our knowledge of these diseases.

In 1934 Fölling found in certain imbeciles in Oslo that there was an excess in the urine of a substance, phenylpyruvic acid. He detected this with the use of a weak ferric chloride solution. It was soon found that the disease, phenylketonuria, was associated with a rare recessive gene in homozygous form. Various physical differences from the normal are present in these children as well as a varying degree of mental impairment. The patients are often blonde, the stature and

FIG. 6.—Sudanophilic diffuse sclerosis. Scharlach R, × 40.

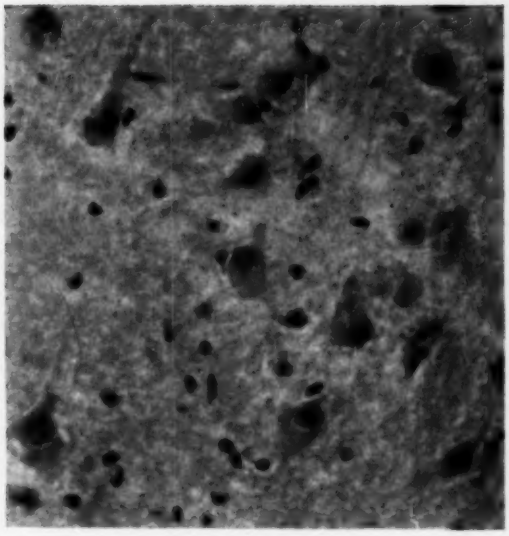

FIG. 7.—Amaurotic family idiocy. P.A.S., × 400.

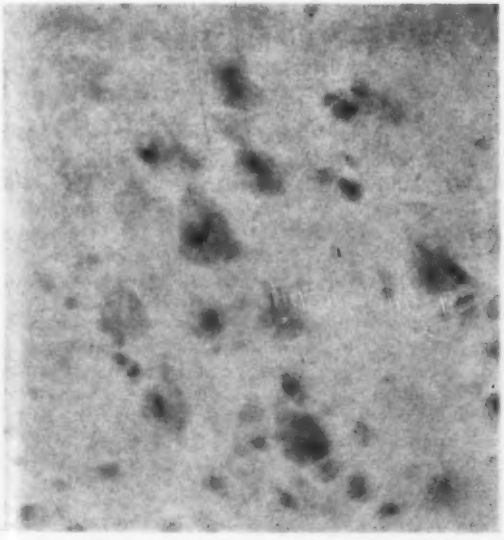

FIG. 8.—Amaurotic family idiocy. Scharlach R, × 400.

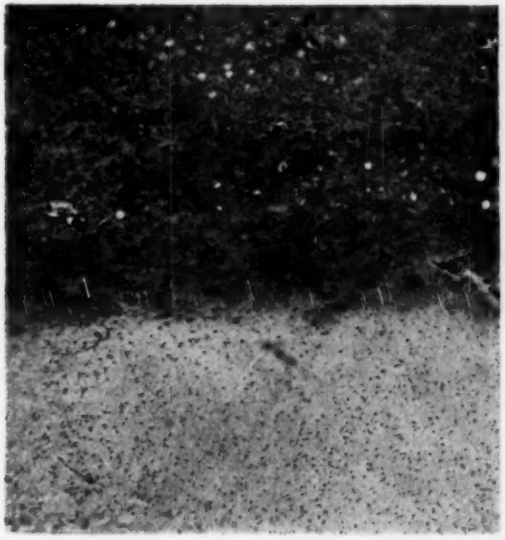

FIG. 9.—Metachromatic leucodystrophy. Toluidine blue, × 40.

head measurements may be reduced, and temperamentally they are docile. The sweat may also be excessive and contain phenylpyruvic acid.

Having discovered a chemical abnormality in the patient, other workers in the past decade proceeded to determine the metabolic pathways that could be involved to produce this abnormality. The fundamental disorder appears to be that the inborn error is one concerned with a failure to metabolize phenylalanine along the normal pathways. In the normal subject phenylalanine is changed in the body into tyrosine by means of an enzyme, phenylalanine hydroxylase. In the patient with this disease this step is not possible, and phenylalanine circulates in the blood and is disposed of as phenylpyruvic acid and phenyllactic acid. There also appears to be an abnormality involving indoles and tryptophane. An attempt has recently been made to treat these patients by dietary restriction of phenylalanine, but this should be done in the first few weeks of life. The patients are given a casein hydrolysate which is freed of phenylalanine, tyrosine, and tryptophane.

Acute porphyria is another condition in which abnormal metabolic pathways have been shown to exist by Rimington. This condition is sometimes associated with a polyneuritis, and I have had the opportunity of seeing and examining a number of such neurological patients.

The third condition I wish to mention is hepatolenticular degeneration, which is now regarded as due to an inborn error of metabolism, for this disease appears to be associated with a disordered copper metabolism. Again the initial observations were made in patients with this disease, for it was discovered by Rümpel that copper and silver appeared to be deposited in the tissues. Although this observation was made in 1913, and further observations along very similar lines were made in 1929 and 1930, it was not until an extensive investigation was made in 1948 that its full significance was appreciated. There is also a considerably increased urinary excretion of copper, and the blood level of copper is low. A substance, caeruloplasmin, which, it has been suggested, may act as a copper transporter, was shown in 1952 by Gitlin and Scheinberg to be absent or only present in minimal amounts in the blood. Two monographs have recently been written on this subject, so that it is obviously impossible to discuss it in full. Further, although some of the chemical abnormalities in the disease have been discovered, the actual mechanism which causes these changes had not yet been found. However, in 1948, as a result of the finding of increased copper deposition, a suggestion was made that B.A.L., a substance mentioned earlier, might be an effective form of therapy. This indeed is the case, for remissions of considerable periods of time have resulted from its use in many patients. Wilson considered that patients with this disease died within four to seven years after the onset of symptoms, and, although older patients have been known to live longer, the younger patients have died sooner. I have investigated 35 patients and personally treated 20 patients with B.A.L., and Table VII shows the results obtained. One of those treated is alive after eight years, and a number lived at least five years. As far as is known, two have married, and a few have been able to work again. Only two are known to have died, and each of these developed a severe cirrhosis of the liver; a portal caval shunt operation was performed and later both died in hepatic coma. The copper content of the liver in both cases, and the brain in one case, was within normal limits, thereby demonstrating the effectiveness of the treatment as far as the copper deposits were concerned. Neurologically, too, they were still improved before death, but the cirrhosis of the liver had advanced relentlessly.

TABLE VII
RESULTS OF INVESTIGATION OF 35 CASES OF HEPATOLENTICULAR DEGENERATION

Copper metabolism, abnormal (blood or urine)	34
Treatment (under personal supervision)	
By B.A.L.	20
,, ,, and penicillamine	11
Follow-up period	
Up to 2 years	8
From 2 to 4 years	9
More than 4	3
Initial improvement after B.A.L.	18
Contact lost with patient	4
Patients died after initial successful treatment	2

This review is very incomplete, only a few subjects having been mentioned, but it may be that it demonstrates the evolving pattern of the biochemistry of the nervous system and the value of basic research when applied to clinical practice.

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THE CLINICAL AND PATHOLOGICAL BACKGROUND OF TWO CASES OF OXALOSIS

BY

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The deposition of calcium oxalate crystals in human tissues was first described by Lepoutre (1925), who found these in the kidney of a child aged 4½ years who also had multiple urethral, ureteric, and bilateral renal calculi. Similar deposits have been described in the kidneys and elsewhere by other authors. Ying Chou and Donohue (1952) suggested the term "oxalosis" to describe the condition in which these crystalline deposits occur, and Scowen, Stansfeld, and Watts (1959) have used it for cases with disseminated extrarenal calcium oxalate deposits. This, though rare, has been identified with greater frequency in recent years. Two further cases are reported here; though they recapitulate many known features of the condition they present some that are new.

Case 1

P. K. was a 5-week-old boy admitted to hospital because he was fretful and refused his feeds. He was a first child and had weighed 7 lb. at birth, which was normal. One week before admission he was weaned from the breast on to dried milk feeds, which he usually took well. During the 24 hours preceding admission he had vomited once and passed a single loose watery stool. His mother had also noticed that he had passed much more urine than usual.

On examination he was pale, listless, and dehydrated, with deep sighing respirations and a temperature of 95° F. There were no other abnormal physical signs.

His blood chemistry was grossly abnormal (Table I), and he was treated with intravenous fluids (1/6 molar sodium lactate and dextrose saline) and antibiotics (penicillin, streptomycin, and terramycin). The day after admission twitching of the left hand and jerky rolling of the eyes were noticed, and in the course of the next few days he developed generalized convulsions, persisting for three days, which were only partly controlled by paraldehyde. Lumbar puncture yielded clear sterile fluid containing 26 cells per c.mm. (mainly lymphocytes) and 50 mg. of protein per 100 ml. During the first four days he passed very little urine (maximum measured volume = 105 ml.), and on the fourth day both kidneys were palpable. Intravenous therapy was discontinued on that day; his urinary output increased thereafter (up to about 500 ml. a day), and he made an uneventful recovery. At this time the serum electrolytes were normal, and when deprived of water he was able to pass a normal concentrated urine.

He was readmitted in coma two months later, his parents having noticed that during the preceding night he had again passed excessive amounts of urine and had been "breathing hard." On examination he was dehydrated with sighing acidotic respirations. His blood chemistry was grossly deranged with the serum bicarbonate 10 mEq./l. on admission. In spite of intravenous therapy he died two days later, aged 14 weeks, no satisfactory diagnosis having been reached.

Investigations.—The following were carried out:

Urine.—Specific gravity was 1011–1016. The urine was acid on admission, becoming alkaline, with a trace of albumin. It was sterile on culture.

TABLE I
BLOOD CHEMISTRY

Date	Sodium	Potassium (mEq./l.)	Chloride (mEq./l.)	Bicarbonate (mEq./l.)	Urea (mg./100 ml.)	Calcium (mg./100 ml.)	Phosphorus (mg./100 ml.)
10/6/58 (i)	160	6·3	122	5	63		
2 hr. later (ii)	160	4·4	116	10			
12/6/58 (iii)	147	4·8	95	6	96	10	9·7
13/6/58 (iv)	156	4·8	104	18			
14/6/58 (v)	140	3·4	90	24	102	8·8	7·7
16/6/58 (vi)	144	3·8	95	27	130	8·6	7·9
10/7/58 (vii)	149	7·9	109	21	30	10·2	5·3
Second Admission							
8/8/58							
7.30 a.m. (viii)	154	7·0	124	10			
11 p.m. (ix)	154	5·4	118	6	87		
9/8/58 (x)	130	5·8	88	10	90	9·4	8·4

Water Concentration Test.—Specific gravity was 1013 after 12 hours' fluid deprivation and 1015 after 18 hours'.

Maximum urea clearance was 31%. The maximum urea concentration was 14.4 g./litre.

An intravenous pyelogram was normal. Paper chromatography of the urine was normal. Rectal swabs grew no pathogens. Over a 17-hour test period three days after admission he excreted only 2 mg. of calcium and 194 mg. of phosphorus in a 135 ml. volume of urine.

Pathology of Case 1 (P.M. No. 284/1958)

The kidneys were slightly swollen and oedematous with a normal pattern and uneven yellow streaking of the medullary pyramids. No calculi were found in the urinary tract. The myocardium was pale, but the heart was normal. The lower lobes of both lungs were slightly collapsed. No abnormality was visible in the remainder of the viscera or in the right femur. There was a small recent subdural haemorrhage (2.5 cm. in diameter) beneath the anterior fontanelle, and a small amount of ante-mortem thrombus in the adjacent sagittal sinus. No visible abnormality in the brain was seen after fixation and hardening in formalin.

The body was that of a pale, well-nourished child with slight pitting oedema of the tissues at the ankles and no evidence of dehydration.

The body weighed 6 kg. (height: 60 cm.), the heart 26 g., spleen 20.7 g., thyroid 1.9 g., testes 2.3 g., suprarenals 4.1 g., pituitary 0.2 g., kidneys (L.) 49.6 g. and (R.) 42.5 g.

Microscopical Examination.—Sections of the pituitary, thymus, thyroid, testis, adrenal, pancreas, liver, spleen, lung, oesophagus, stomach, mesenteric lymph node, large and small intestine, kidneys, and brain were examined, but abnormalities were confined to the last two organs. Unfortunately the bones were not examined because the nature of the disease was not realized at the time of necropsy.

Kidneys.—The renal tubules contained many pale, greenish-yellow doubly refractile crystals, yielding green, yellow, and orange colour patterns in polarized light. These were mostly arranged as broken plates, or, more commonly, as radiating sheaves forming rosettes (Fig. 1). These crystalline deposits distended and distorted the lumina of the tubules, particularly the distal convoluted segment. There were patchy areas of necrosis in the proximal convoluted tubules, and degeneration of the tubular cells in other areas with swelling, disruption, and a rather foamy appearance of their cytoplasm. There was haemorrhage into some of the tubules, particularly proximally. The glomeruli, vessels, and interstitial tissue were normal. There was remarkably little inflammatory response to the crystalline foci, though occasionally they were partly enclosed by giant cells. Only rarely were the tubules actually disrupted and there was virtually no destruction of the normal microscopic renal architecture (Fig. 2).

Brain.—Similar crystals were found unevenly distributed in the brain and cerebral vessels. In the brain substance they were most numerous in the hypothalamus and convolutional white matter, though scanty deposits were present in the cerebellar white matter and in the medulla. The choroid plexus contained none. In some of these sites they had produced no reaction, but a rosette of microglial cells surrounded occasional groups (Fig. 3a). Many of these deposits were paravascular in relation to capillaries, but in the leptomeninges crystals occupied the media of a few small arteries (Fig. 3b). Areas of focal meningitis, with infiltration by polymorphs and large round cells, were found over the floor of the third ventricle and in the depths of a few cerebral sulci, and in the choroid plexus of the left lateral ventricle. These bore no relation to the crystalline deposits, and no bacteria were demonstrated. The solubilities and staining properties of these crystals, together with their chemical composition, are discussed below.

Case 2

D. R. was the only son of a first-cousin marriage. At the age of 8 he passed a stone of unknown composition per urethram, but had no other urinary troubles. At the age of 11 he developed painful raised red swellings on both shins that persisted for about two weeks, which were regarded as erythema nodosum.

Nearly two years later he developed a cough producing frothy white sputum, after which he became increasingly breathless on exertion. Six months later he was admitted to another hospital on account of progressive oedema of the ankles, legs, and abdomen. He was then found to have signs of constrictive pericarditis with bilateral pleural effusions, which were tapped. Treatment was started with antituberculous drugs (streptomycin and I.N.A.H.) and diuretics (neptal and diamox). One month later, on admission to the London Hospital, he had signs of constrictive pericarditis with pitting oedema to the waist, an enlarged liver, raised jugular venous pressure, and persistent bilateral effusions. An E.C.G. showed changes compatible with pericarditis. Treatment was continued with the same antituberculous drugs, diuretics (diamox), and a restricted salt intake. At this time the blood urea level was 37 mg./100 ml. A week after admission treatment was begun with

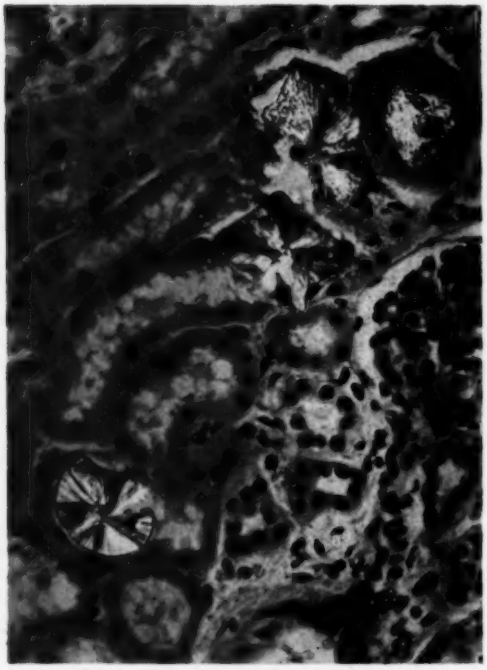


FIG. 1.—Kidney from Case 1. Doubly refractile rosettes of crystals occupy tubules. Haematoxylin and eosin, ×350. Photographed in partly polarized light.

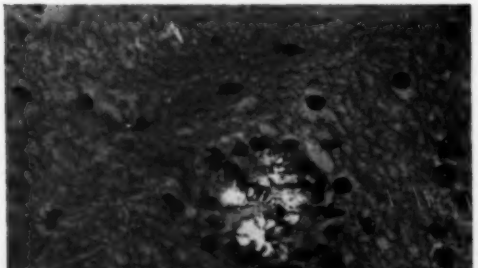


FIG. 3a.—Brain from Case 1. Doubly refractile crystals in hypothalamus enclosed by microglial cells. Haematoxylin and eosin, ×240. Partly polarized light.

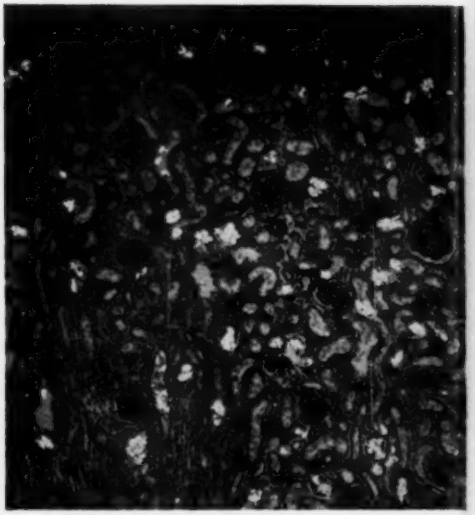


FIG. 2.—Kidney from Case 1. Many doubly refractile crystals in tubules, but none in glomeruli. Normal renal architecture well preserved. Haematoxylin and eosin, ×60. Partly polarized light.

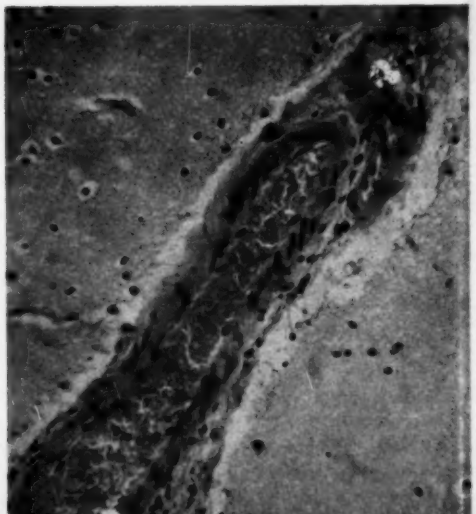


FIG. 3b.—Brain from Case 1. Crystals in the media of small meningeal artery, evoking no reaction. Haematoxylin and eosin, ×400. Partly polarized light.

FIG. 4a.—Plain radiograph of abdomen of Case 2, showing bilateral renal calculi.

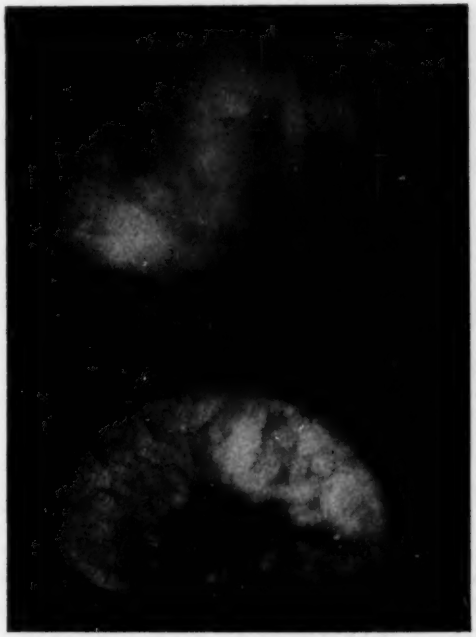


FIG. 4b.—Radiograph of kidney from Case 2 (below) removed at necropsy compared with control.

digoxin and chlorothiazide, which produced a good diuresis and a steady diminution in the oedema. During the next month, however, he became febrile and developed a right-sided pleural friction rub. The E.S.R. rose, and a chest radiograph showed an area of consolidation at the apex of the right lower lobe. A tuberculous "flare-up" was suspected and P.A.S. started. Ten weeks after admission the blood urea level was first raised (84 mg.%), and renal function was further investigated. A straight radiograph of the abdomen showed bilateral renal calculi (Fig. 4a) but no obvious nephrocalcinosis. (A post-mortem radiograph, however, showed fine radial streaking (Fig. 4b).) Meanwhile he slowly deteriorated and the blood urea rose steadily, until six months after admission it reached 260 mg.%. In an attempt to relieve any possible obstruction right ureterolithotomy was performed a few days later, and a calcium oxalate stone was removed from the right pelvi-ureteric junction. A renal biopsy was also taken. Post-operatively his urinary output declined, his blood pressure fell, and he died 54 hours later.

Investigations.—The following investigations were carried out:

Blood.—Haemoglobin was 96%, falling to 56%. A white cell count gave 5–11,000 with regularly 70–80% polymorphs. The E.S.R. was 7–45 mm./hour. The serum calcium level was 11.7, 10.1, 11.4, 6.9, and 7 mg.%, and the serum phosphorus level 6.1, 5.2, 5.3, 7.4, and 7.5 mg.%, and alkaline phosphatase 8, 14, 25, and 27 King–Armstrong units. (The last two estimations in each case were carried out within three weeks of death.) Other serum electrolytes were normal, except for some variation in potassium (3.8–7.5 mEq./l.) due to the administration of chlorothiazide with potassium supplements.

Blood Urea.—On February 7 the level was 20 mg.%; between March 2 and April 2, 33–37 mg.%; on May 15, 84 mg.%, after which it rose steadily to reach 260 mg.% on September 4 (13 days before death).

Liver Function.—Tests were repeatedly normal, except for a single raised bilirubin value of 1.3 mg.% at the time when he had the fever, friction rub, and raised E.S.R. Plasma proteins were 5.6 g.% rising to 7.5 g.% (albumin 5.1, globulin 2.3).

Urine.—Specific gravity never rose above 1012, except on one occasion after a course of chlorothiazide. Urine was sterile on culture, containing a trace of albumin and a moderate number of white blood corpuscles. Paper chromatography and 24-hour cystine excretion were normal. Calcium-balance studies were carried out a month before death, but their results must be interpreted with caution as the boy was ill, vomiting, and in renal failure by then.

However, with an intake of 543, 639, and 612 mg. of calcium on three successive days the urinary excretion was only 18, 37, and 21 mg. of calcium respectively. A single specimen of urine (175 ml.), which was virtually his total output in his last 24 hours, contained 23 mg. anhydrous oxalic acid per 100 ml.

No tubercle bacilli were identified or cultured from sputum, pleural aspirate, or urine.

Pathology of Case 2 (P.M. No. 313/1958)

The capsule was slightly adherent to the smooth surface of the kidneys, which were of normal size for the boy's age. Numerous fine yellow gritty flecks were seen on the outer surface and throughout the cut surfaces, especially in the cortex (Fig. 5). The pattern was not obviously blurred, and the calices, pelves, and ureters were not dilated. No calculi were found in the urinary tract. There was a sutured incision in the lower pole of the right kidney. The parietal pericardium showed a slight fibrous thickening (0.1 to 0.2 cm.), producing stenosis (to 1.5 cm. in circumference) of the superior vena cava at its junction with the right atrium. The pericardial sac was obliterated by fibrous adhesions, separable posteriorly but not anteriorly. No evidence of tuberculosis was found. There was no visible abnormality of the heart. Slight fibrous thickening of the visceral pleura was visible with delicate adhesions over the postero-inferior surfaces of the right lower lobe, in which there was a recent infarct in the upper posterior segment. Patchy congestion was seen throughout both lower lobes. There was no evidence of active or obsolete tuberculosis, apart from focal calcification of a lymph node eroding the trachea on the right, 2 cm. above the carina. About 12 superficial ulcers were found in the rectal mucosa, but the remainder of the alimentary tract was normal. The right femur was slightly bowed, with normal dense cortical bone (up to 0.6 cm. thick) and reddish-brown marrow in the upper two-thirds of the shaft and head and dense cancellous bone at its extremities and in the vertebral bodies. Colloid was abundant in the nodular, slightly enlarged thyroid. Three unenlarged parathyroid glands were found at normal sites. The nervous system was normal.

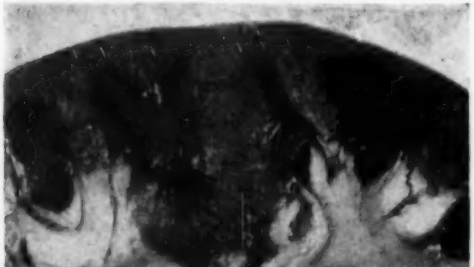


FIG. 5.—Photograph of part of cut surface of kidney from Case 2 to illustrate finely granular radiate yellow streaking (cf. Fig. 4b).

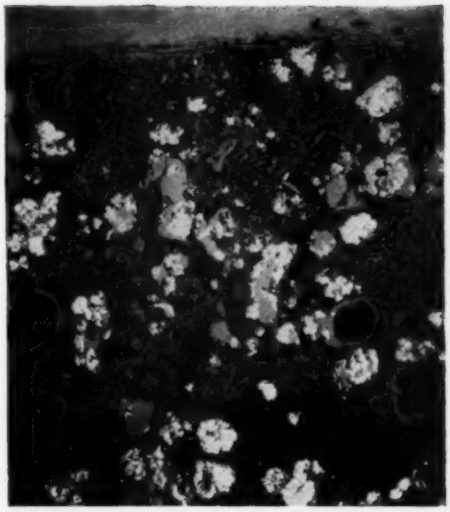


FIG. 6.—Kidney from Case 2. Doubly refractile crystals in tubules and interstitial tissue, but glomeruli again spared. Considerable disruption of normal renal pattern. Haematoxylin and eosin, ×60. Partly polarized light.

The body was that of a poorly nourished, slightly built boy, with brownish pigmentation of the external genitalia and extremities and a recent sutured incision in the right loin.

The body weighed 40.2 kg. (height: 163 cm.), the heart 269 g., spleen 134 g., thyroid 17.8 g., testes 18.1 g., suprarenals 11.4 g., pituitary 0.4 g., each kidney 92 g.

Microscopical Examination.—In this case similar doubly refractile crystals were found rather more widely distributed throughout the body.

Kidneys.—The renal architecture was distorted (Fig. 6), the tubules distended and disrupted by crystalline deposits which were also found lying in rosettes only partly surrounded by tubular cells, and in some instances even in the interstitial tissue. There was widespread degeneration of the tubular epithelium which was markedly thinned in some of the more dilated tubules, and haemorrhage into the tubules, some of which contained granular

casts. Although there seemed to be little reaction to the crystals there were scattered foci of fibrosis and round cell infiltration, particularly in the cortex. The glomeruli and vessels again contained no crystals and appeared normal.

Bones. — Similar crystalline deposits also occurred in vertebrae, femur, and sternum. In the first these lay mainly in lacunae at the osteochondral junction (Fig. 8a) where they were often related to active foci of osteoblasts. A few were found in the bony trabeculae, and this was their site in the sternum. They had produced no reaction and no obvious disturbance of bone growth. None were found in the marrow at any of these sites. The thyroid gland contained occasional crystals, both in the follicles and interstitial tissue, whilst the testis contained crystals within the lumina of some of the seminiferous tubules (Fig. 9). In none of these sites did they evoke any cellular reaction. No other endocrine glands contained crystals. The three parathyroids were normal. There was a mass of similar crystals in the paratracheal lymph node which was eroding the trachea, particularly concentrated around necrotic calcified material (Fig. 10). A carinal and mediastinal lymph gland contained no crystals. Occasional doubly refractile crystals were seen within some of the cardiac muscle fibres, whilst there were scanty deposits in the fibrotic pericardium. The latter contained a few foci of lymphocytes. Sections of the lower lobe of the right lung showed oedema with an area of haemorrhagic infarction and foci of early purulent bronchopneumonia. No tubercle bacilli, giant cells, epithelioid cells, or caseation were found in the lungs and pericardium. No crystals were found in sections of liver, spleen, pancreas, or rectum. Unfortunately the brain was not examined.

Identification of Crystalline Material

Solubilities and Staining Reactions of the Crystals.—In each case the crystalline deposits were observed in paraffin sections prepared in the routine way after fixation with 4% saline formaldehyde. They were insoluble in dilute and glacial acetic acid, saturated ammonia, and 10 N potassium hydroxide, but were readily soluble in dilute mineral acids, e.g., normal hydrochloric or sulphuric. They did not stain by haematoxylin, eosin, methyl or cresyl violet, and Schmorl's reagent. Both Alcian blue and Schiff's reagent (containing dilute hydrochloric acid) dissolved the crystals but stained amorphous material at the same sites, possibly indicating some organic crust or matrix associated with them. Sections containing crystals decolorized dilute warm solutions of potassium permanganate unlike control preparations, and after exposure to acid permanganate the crystals disappeared. The crystals were stained black by the von Kossa method (Fig. 7). All these properties lead to the conclusion that the crystals are calcium oxalate.

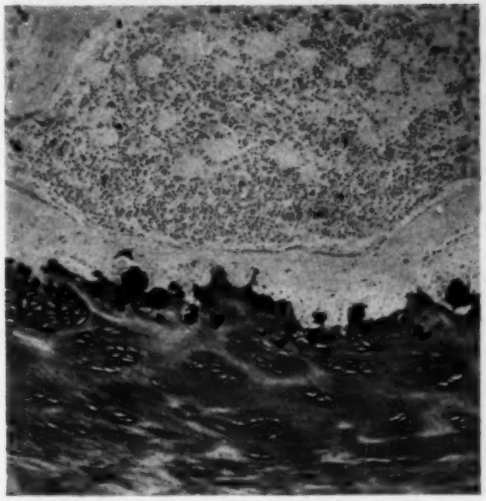


FIG. 7.—Vertebra from Case 2. Deposits stained black by von Kossa method, ×77.

Chemical Extraction of Kidneys.—The oxalic acid and calcium contents of the kidneys of the two cases were estimated. The procedure for estimating oxalic acid was somewhat complex, being a modification of that devised by Andrews and Viser (1951) to estimate the oxalic acid content of food. It depends on successive extractions of the test material with acid and ether.

These authors studied the effect of altering the concentration of hydrochloric acid and the time of extraction in the first stage, and the acidity and time of ether extraction in the second. They concluded that extraction for five hours with a concentration of HCl between 3–7 N, followed by extraction with ether for 15 to 25 hours, was the most effective method.

Method.—Whole kidneys were stripped of their capsules and pelvic fat and minced to a fine powder, which was dried to constant weight. Aliquots of approximately 2 to 5 g. were then placed in a coarse sintered glass crucible and extracted for five hours with 6N HCl in a Soxhlet extractor. This extract,

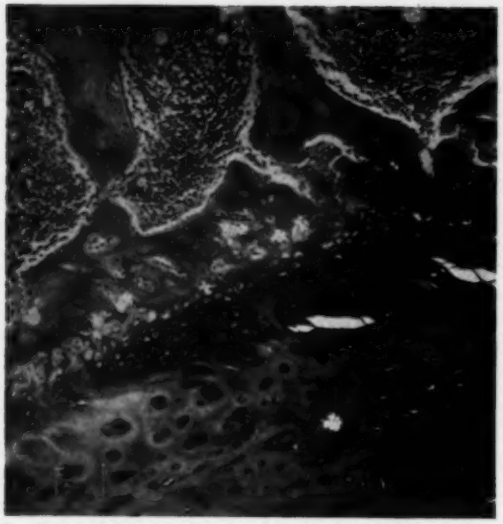


FIG. 8a.—Vertebra from Case 2. Deposits at osteochondral junction and in trabecular bone. Haematoxylin and eosin, ×66. Partly polarized light.

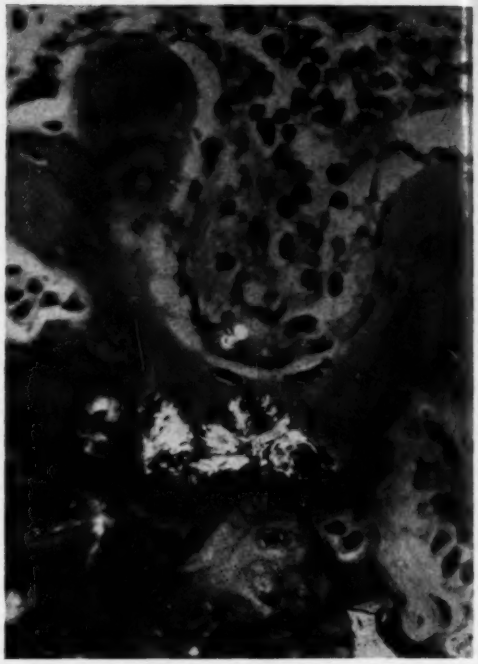


FIG. 8b.—High magnification (×370) of Fig. 8a, showing crystals in trabecular bone.

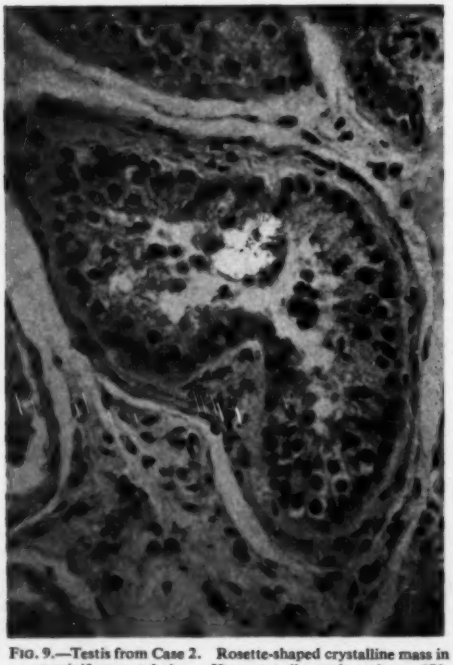


FIG. 9.—Testis from Case 2. Rosette-shaped crystalline mass in seminiferous tubule. Haematoxylin and eosin, ×270. Partly polarized light.

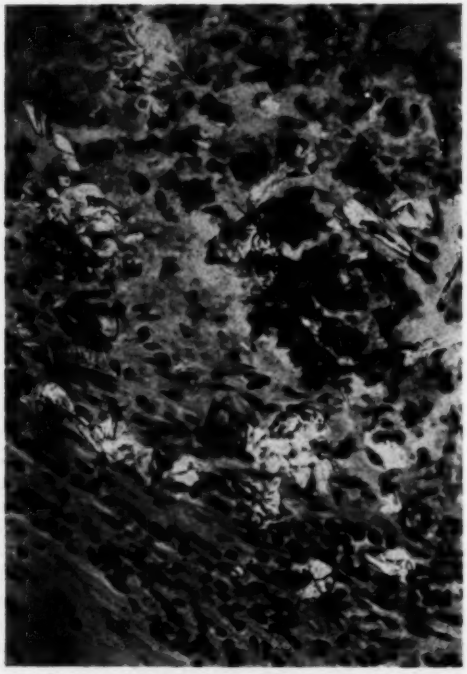


FIG. 10.—Paratracheal lymph gland from Case 2. Many doubly refractile crystals close to deeply staining necrotic calcified material. Haematoxylin and eosin, ×330. Partly polarized light.

together with washings from the apparatus, was allowed to cool and then filtered through a Whatman's 42 (acid-resistant) filter paper. Aliquots, each of 30 ml., of this filtrate were then extracted with ether (using 200 ml. ether and 25 ml. of distilled water) for 20 hours in a liquid-liquid extractor. The ether was distilled off and the resulting aqueous solution was adjusted to *p*H 5.2 as recommended by Archer, Dormer, Scowen, and Watts (1957a) and the volume made up to 80 ml., which was filtered and decanted into two 50 ml. centrifuge tubes.

Excess $CaCl_2$ was added to precipitate any oxalic acid as calcium oxalate, and the tubes left standing for 16 hours. At the end of this time a grey precipitate formed. The tubes were spun, the supernatant fluid removed, and the precipitate washed with falling dilutions of ammonia (10N, N, and finally 0.35N). The resulting white precipitate was dissolved in N H_2SO_4 and titrated with standard permanganate at 60°. The results were expressed as oxalic acid dihydrate $(COOH)_2\,2H_2O$.

Calcium was estimated by ashing the dry kidney extracts to calcium carbonate, which was then dissolved in acid. The solution was rendered alkaline with ammonia and excess ammonium oxalate added. The resulting precipitate was washed and titrated as above.

Results.—The results are shown in Table II. A few points arise. First, a small amount of N/100 permanganate was needed to produce lasting pink coloration of solutions of N H_2SO_4. This was taken as a blank reading. After final washing of the controls there was no appreciable precipitate left, though the solution was faintly cloudy. This cleared on the addition of acid and a minute amount of N/100 $KMnO_4$ (0.01–0.02 ml.) above the blank reading was needed to give a lasting pink colour. Even taking this into account, the values were less than 0.1 mg. oxalic acid per gramme dry weight of kidneys for all three controls. On the addition of 50 mg. of oxalic acid to one control kidney, 98.2% recovery was obtained. This is not a perfect control, but seemed the most practical in the circumstances. The results revealed large amounts of calcium and oxalic acid in the two affected kidneys, the total oxalic acid content of the kidney of Case 2 being over 1 g.

TABLE II

CALCIUM AND OXALIC ACID CONTENTS OF KIDNEYS

Kidney	Dry Weight (g.)	Oxalic Acid ($(COOH)_2\,H_2O$) (mg./g. Dry Weight)	Calcium (mg./g. Dry Weight)
Control (1)* ..	12·72	Nil†	
,, (2) ..	13·85	Nil	0·13
,, (3) ..	6·28	..	0·05
Case (1)	5·13	18·9	12·5
,, (2)	13·45	77·2	57·0
Control (2)+50 mg. oxalic acid ..		49·5	

* Controls were: (1) man of 57 with mesenteric thrombosis; (2) man of 52 with bronchial carcinoma; (3) boy of 6 months with gastro-enteritis.

The control kidneys, which approximated in dry weight to those of the two cases, were removed at necropsy and immediately dried to constant weight. Sections of each were examined and contained no crystals.

† See text.

X-ray Diffraction Studies.—The crystalline deposits in kidneys from Cases 1 and 2 were identified as calcium oxalate monohydrate by *x*-ray diffraction studies kindly carried out by Dr. R. J. R. Cureton, of St. Bartholomew's Hospital.

Discussion

Review of Cases Already Reported.—Although this paper primarily reports the findings at necropsy in two cases of oxalosis, the nature of this condition and its relation to oxalate metabolism will be considered.

Calcium oxalate crystals were first identified in the urine by Donné in 1839. This led, during the next 50 years, to the concept of an "oxalic acid diathesis," which consisted of the association of large numbers of such crystals in the urine of patients with various dyspeptic, nervous, and rheumatic symptoms (Bird, 1842; Begbie, 1849), and received much attention from French workers, who even compared oxaluria with gout, coining the phrase "*goutte oxalique*" (Loeper, 1933) which, besides its rheumatic manifestations, could present with anaemia, migraine, and even intestinal "crises" similar to those of tabes. Raised levels of oxalic acid in the blood, urine, and cerebrospinal fluid (Rodillon, 1933) were described, but the methods of estimating oxalate were by no means perfect, and many of the results were conflicting. After about 1900 the "oxalic acid diathesis" fell into disrepute in English- and German-speaking countries, although still described in the medical textbooks (Osler, 1935), and interest in it has been renewed only in the last decade. This has been due to the development of more satisfactory methods of determining oxalates (Barrett, 1942; Archer *et al.*, 1957b), and to the description by various authors (Zollinger and Rosenmund, 1952; Burke, Baggenstoss, Owen, Power, and Lohr, 1955, etc.) of deposits of calcium oxalate crystals at various sites in the body, particularly the renal tubules. In the last 10 years over 20 cases of oxalosis have been reported. In all these calcium oxalate crystals were found in the renal tubules, also at other sites in some. Some of these cases are compared and contrasted in tables by Dunn (1955) and by Archer *et al.* (1958a). Haematuria, with or without the passage of calculi, was the principal

clinical feature. Nephrocalcinosis, nephrolithiasis, or both, formed a constant radiological finding.

Most patients died in childhood or early adult life, the ages of death ranging from 5 months to 22 years. Four cases dying in middle life, the eldest aged 57, have been reported, however (Zollinger and Rosenmund, 1952; Lund and Reske-Nielsen, 1956). Death in all cases was due to renal failure, with or without complicating urinary infection. In many cases the diagnosis was only reached in retrospect at necropsy, but in four cases (Aponte and Fetter, 1954; Godwin *et al.*, 1958; Scowen *et al.*, 1959) urinary oxalate estimations *in vivo* had previously established an increased excretion of oxalic acid (hyperoxaluria). Archer *et al.* (1957a, 1958a) have suggested that the triad of hyperoxaluria, nephrocalcinosis or nephrolithiasis, and oxalosis forms a single syndrome (" primary hyperoxaluria "), which may be an inborn error of metabolism, comparable with cystinuria and xanthinuria and perhaps hereditary. In support of this hypothesis, Aponte and Fetter (1954) reported cases in twins and in a third sibling, whilst Newns and Black (1953) suspected the condition in the brother of their case; also Edwards (1957) has reported crystalline deposits of calcium oxalate in the renal parenchyma and myocardium of two brothers dying of renal failure. The father of Dunn's case had passed a single calculus of unknown composition. Myers (1957) mentions three cases of suspected oxaluria occurring in twin girls and their sibling brother. Archer *et al.* (1958b), however, carried out urinary oxalate determinations on members of three families containing cases of hyperoxaluria, but failed to find an increased oxalate excretion in any of the related subjects.

Since, however, each component of the triad can occur independently the finding of a single one may be insufficient to establish the diagnosis. As pointed out by Archer *et al.* (1958a), an increased oxalate excretion is the most reliable criterion. But hyperoxaluria has been described in other conditions. Thus Baldwin (1900) described it in dogs following prolonged feeding with excessive amounts of glucose, whilst Loeper (1933) and others (Jeghers and Murphy, 1945) claim that it is often associated with diabetes. Hyperoxaluria has also been reported following the administration of such compounds as gelatin, glycerin, creatine, and purines (Barber and Gallimore, 1940), and ascorbic acid (Lamden and Chrystowski, 1953). It also occurs in ethylene glycol poisoning (*vide infra*).

Although most cases of hyperoxaluria have calcium oxalate stones (usually pure) in the renal tract the reverse is far from true. Calcium oxalate, either pure or mixed with phosphate, carbonate, or urate, is a common constituent of urinary calculi. The percentage of urinary calculi reported to contain oxalate varies from author to author and from country to country. In America over 30% of stones contain oxalate (Jeghers and Murphy, 1945), whilst in central Europe between the wars it was reputed to be 65–90%. As hyperoxaluria often presents in childhood, the observation by Myers that 12 out of 59 urinary calculi (approximately 20%) analysed at The Hospital for Sick Children, Great Ormond Street, between 1936 and 1956 contained calcium oxalate is perhaps more pertinent. Even oxalate calculi in children, however, can exist independently of hyperoxaluria as was shown when Archer *et al.* estimated the urinary oxalate excretion of the three siblings and other members of the family mentioned by Myers, all of whom were found to have a normal oxalate excretion. Recently Hodgkinson (1958) has measured the 24-hour urinary excretion of oxalic acid in 19 subjects with pure or mixed calcium oxalate stones and found it to be raised in a single instance only. The pathogenesis of calcium oxalate calculi is obviously complex, and the excretion of excessive amounts of oxalic acid is but one factor. Further, the tendency to form such stones may be hereditary. Gram (1932) published the pedigree of his own family, a number of whom (15 in five generations, all male) had suffered from renal calculi. These had been analysed and found to contain calcium oxalate in five cases. From his data he suggested that the disposition to form oxalate urinary calculi might be inherited as a single dominant character, only clearly showing itself in males (possibly due to such factors as urinary stasis behind an enlarged prostate). Only one of his cases had symptoms before the age of 30, however, and no urinary oxalate estimations were carried out. It cannot therefore be claimed that this family had hyperoxaluria, although a strong case is presented for a hereditary tendency towards oxalate stone formation. There are, of course, many known causes of nephrocalcinosis besides primary hyperoxaluria.

Oxalosis can also occur as a separate entity. It was found incidentally by Laas (1941), who described calcium oxalate crystals in the renal tubules of a man of 25 who died of peritonitis after a gunshot wound. Glynn (1940) found doubly refractile crystals arranged in a rosette

pattern in the middle cerebral artery of a woman of 61 dying from rupture of an aneurysm of the left middle cerebral artery. From their solubilities and staining reactions he concluded that these were calcium oxalate. Doubly refractile calcium oxalate crystals have also been described in the thyroid gland by Richter and McCarty (1954) and others. The significance of these sporadic deposits is uncertain.

The Present Cases.—Although the output of urinary oxalate was not estimated in the two personal cases described in this paper, the condition of oxalosis was established by histological examination and the diagnosis of primary hyperoxaluria appears warranted.

Case 1.—This is the youngest fatal case so far reported. The clinical picture here of extreme acidosis and severe dehydration provided a unique clinical problem in a different category from the "hyperchloraemic acidosis" described by Lightwood (1935) or by Butler, Wilson, and Farber (1936). These last authors described four fatal cases in children under the age of 1, presenting as persistent dehydration and acidosis in the absence of vomiting, diarrhoea, or inadequate fluid intake. These infants had a raised plasma chloride (up to 132 mEq./l.) and reduced bicarbonate (down to 6 mEq./l.). At necropsy calcium was deposited in the walls of the renal tubules, but no doubly refractile crystals were found. In Case 1, however, once the initial dehydration was overcome the low bicarbonate persisted with a normal chloride level (Table I). Another interesting point is the delayed diuresis and the persistent elevation of the blood urea after the acidosis had been corrected.

The cause of the convulsions is uncertain, though the two other cases reported in children under 1 year (Mulloy and Knutti, 1951 ; Katzuni and Sandbank, 1959) also suffered from them. It has been suggested (Jeghers and Murphy, 1945) that such convulsions could be due to hypocalcaemia, and the serum calcium was, in fact, at one time reduced to less than 10 mg. per 100 ml. They were more likely due to the presence of an increased concentration of oxalate in the central nervous system. In the present case and in that reported by Katzuni and Sandbank, calcium oxalate crystals were found in the brain, and the cerebrospinal fluid in the case reported by Scowen *et al.* (1959) contained appreciable amounts of oxalate. That oxalates can damage the brain and cause convulsions is also suggested by the findings in cases of ethylene glycol poisoning. Pons and Custer (1946) described 18 fatal cases, most of which died within 24 to 48 hours after convulsions and coma. At necropsy calcium oxalate crystals were found in the renal tubules, brain substance, and cerebral blood vessels. These cases also showed changes in the brain suggestive of sterile meningitis or encephalitis. In fact the microscopic findings in the brain closely resembled our findings.

The changes in the kidneys are evidently those of an uncomplicated oxalosis, the crystals being confined to the tubules and the normal renal architecture being well preserved. Most of the cases previously reported have been in older subjects, many of whom showed additional renal changes secondary to prolonged pyelonephritis. In fact the changes in Case 1 are similar to those found in rabbits with induced "oxalate nephritis."

Dunn, Haworth, and Jones (1924a) studied the effect of administering soluble oxalate salts to rabbits, and found that when moderate doses were given for prolonged periods (four to 25 days) lesions in the proximal renal tubules were found at necropsy. These included a foamy appearance in the epithelial cells and desquamation of cells into the lumina, which were packed with calcium oxalate crystals. The glomeruli were normal, as were the ascending loops of Henle and second convoluted tubules, apart from some dilatation. No mention was made of oxalate deposits elsewhere in the body. Macroscopically the kidneys were pale and swollen, with cut surfaces finely marked with yellow flecks in the natural pattern of the renal tubules (cf. Cases 1 and 2). In further studies (Dunn *et al.*, 1924b) of experimental "oxalate nephritis" they found that on administering oxalates there was an initial oliguria, later followed by polyuria. Albuminuria was a constant finding, associated with an increasing elevation of the blood urea and the passage of pale dilute urine. These features, which cleared up when oxalates were withdrawn, have much in common with those observed in Case 1.

Case 2.—It is noteworthy that the parents of this boy were first cousins. Unfortunately he was an only child, and it was not possible to investigate other members of his family. His rapid deterioration is a clinical puzzle. Although the constrictive pericarditis may have played a part, it is also possible that the induced diuresis may have promoted the precipitation of calcium oxalate in the renal tubules. In support of this idea, it will be recalled that in Case 1 the two acidotic episodes were preceded by diuresis.

Pathologically the sites of crystal deposition are intriguing. The most commonly reported sites of deposits are in kidney, bone, and myocardium,

whilst other sites include thymus, testis, and pancreas (Neustein, Stevenson, and Krainer, 1955), thyroid and adrenals (Burke *et al.*, 1955). More recently crystals have been reported in the pulmonary alveoli and lymph nodes (Godwin *et al.*, 1958), whilst Scowen *et al.* (1959) described them in many of these sites and also in arteries and arterioles throughout the body; also sparsely in the anterior pituitary, tonsil, and ciliary body of the eye. Perhaps the most interesting sites in Case 2 are at the osteochondral junction of bone and in a partly calcified lymph gland, for here a high concentration of calcium ions would be expected and would be available to combine with oxalic acid. The presence of deposits in arterial and arteriolar walls and in myocardium remains inexplicable, though Scowen *et al.* (1959) have suggested an association with smooth muscle metabolism.

Various authors have reported the calcium and oxalate contents of kidneys in affected cases, but many of these values have been expressed as percentages of wet weight of kidney and the method of determination has not been described. The values in Case 2 are of the same order as those reported by Scowen *et al.* (1959), using the same method.

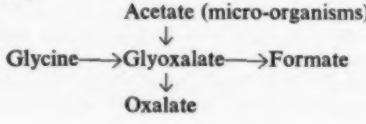
The relationship to calcium metabolism is uncertain, but, in the limited investigations carried out, both cases had a reduced calcium excretion, while the serum calcium in Case 2 declined terminally.

Possible Aetiology.—The work of Scowen's group (Scowen, Crawhall, and Watts, 1958) and others suggests that the excretion of excessive amounts of oxalate in primary hyperoxaluria is due to abnormal endogenous formation, rather than to any excessive absorption from the gastro-intestinal tract or a low renal threshold for oxalate. The exact biochemical lesion responsible is unknown, however. Oxalic acid has no known role in mammalian intermediary metabolism, and indeed some experiments suggest that it is relatively inert (*vide infra*). In some moulds it is formed from acetate with glycolate and glyoxalate as intermediaries (Nord and Vitucci, 1947). Weinhouse and Friedmann (1951), however, using C^{14}-labelled compounds, failed to demonstrate such a pathway in live rats, although glyoxalate was certainly converted to oxalate. Moreover, oxalic acid seemed relatively inert as it was converted neither to glyoxalate nor to CO_2, and 80% of the administered dose could be recovered from the urine and tissues. The administration of ethylene glycol, glyoxal, glycolic acid, purines, and creatinine has been reported to increase oxalate excretion in animals (Jeghers and Murphy, 1945), but it has been impossible to demonstrate oxalate synthesis by mammalian tissue slices from most of these substrates, nor was oxalate destroyed by such slices (Barber and Gallimore, 1940). In rat liver, however, the reaction glycine⟶glyoxalate ⟶formate has been demonstrated by Nakada, Friedmann, and Weinhouse (1955), and Nakada and Weinhouse (1953) have shown that when glyoxalate is present in excess it is converted to oxalate, thus providing a possible pathway for oxalate formation from glycine.

Experiments by Scowen's group (Archer *et al.*, 1958a) have, in fact, suggested that oxalate arises from glycine in man; for the oxalate excretion of patients with primary hyperoxaluria was reduced by lowering the free glycine metabolic pool by giving sodium benzoate. In a further study (Scowen *et al.*, 1958) the administration of glycine-1-13C led to considerable incorporation of the isotope into the urinary oxalate.

Yet another possible source of glyoxalate is from acetate via the "glyoxalate cycle" recently described by Kornberg and Krebs (1957). But this has so far only been established in micro-organisms.

The evidence at present favours glyoxalate as an immediate precursor of oxalate, this in turn arising from glycine or, less likely, from acetate. On this scheme excessive glyoxalate could arise as a direct result of excessive formation from glycine (or acetate), or alternatively by blocking of the reaction glyoxalate⟶formate, or one of the normal metabolic pathways of glycine so that more glycine was metabolized along the glyoxalate-formate pathway (see diagram below). Since the condition appears to be hereditary such a metabolic block could be a manifestation of a genetically transmitted enzyme deficiency, as occurs in the "inborn errors of metabolism" (Garrod, 1923). Further studies are obviously needed, but unfortunately the biochemical investigation of this condition is difficult: first, the disease is rare; secondly, there is no satisfactory method to estimate blood oxalate levels (Barrett, 1943); and, thirdly, many cases are in advanced renal failure when diagnosed, so that studies of their oxalate excretion must be interpreted cautiously.



Crystal Formation.—The condition poses other problems. The sites of crystal deposition have already been discussed, but why do they form rosette patterns in the body quite different from the form taken on crystallization out of simple solution? Their shape, which is similar to that found in some plants (Glynn, 1940), may indeed be due to their precipitation in some sort of organic matrix as suggested by their staining reactions (*vide supra*). Indeed, it has been suggested (Keyser, 1938) that calcium oxalate crystals agglutinate in an organic framework of colloid to form calculi. How rapidly do the crystals form and what factors initiate their formation? Crystals are found within 24 hours in cases of ethylene glycol poisoning, suggesting a rapid formation, but on the other hand nephrocalcinosis has been observed some time before death. In fact, the speed of deposition of crystals and the extent to which they impair renal function may vary, and crystals may be precipitated in definite periodic attacks, during which the patient may succumb or survive. Several of the cases reported give a history of "attacks" apart from those due to calculi, and Case 1 survived his first attack. It is likely that oxalate excretion is increased at such times with the precipitation of calcium oxalate in the renal tubules. In Case 1 the massive deposition of oxalate crystals in the renal tubules caused death, but in other cases calculi rather than crystals may form, leading to recurrent calculous pyelonephritis. Death from renal damage may be due to this rather than to damage from the crystals themselves, as suggested by Scowen *et al.* (1959) in their cases. The cause of these episodes of crystal deposition remains uncertain. Dehydration may be a factor, and Black (1945) has suggested, on the basis of the finding of oxalate crystals in the urine, that episodes of such crystalluria due to dehydration were responsible for many cases of renal colic and haematuria among British troops in India in the last war.

Summary

Two fatal cases of oxalosis are presented: the first was in a boy of 14 weeks who presented with severe dehydration and acidosis and had crystalline deposits of calcium oxalate in the renal tubules and brain. Attention is drawn to this method of presentation, as it seems possible that if it is borne in mind further cases may be diagnosed.

The second case was in a boy of 14 years who had bilateral renal calculi and rapid renal failure. Here crystals were found in kidneys, bone, myocardium, thyroid, testis, and calcified lymph node.

The pathogenesis of the condition remains uncertain, but the evidence so far strongly suggests that an inborn error of metabolism leads to excessive oxalate formation.

It is a pleasure to thank Professor Dorothy Russell for her constant encouragement and guidance, Dr. R. H. Dobbs and Dr. Wallace Brigden for permission to publish the clinical details of cases under their care, and Dr. R. W. E. Watts, of St. Bartholomew's Hospital, for advice in the chemical estimations; also Mr. J. King for the photomicrographs.

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J. clin. Path. (1959), **12**, 510.

THE PROTEINS OF NORMAL URINE

II. FROM THE URINARY TRACT *

BY

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Normal urine contains traces of many different proteins. These proteins include:

(1) Plasma proteins entering the urine from the blood through the glomeruli.

(2) Urinary tract proteins from the glands or shed cells of the urinary tract from the kidney tubules downwards. One protein, the mucoprotein of Tamm and Horsfall (1952), has been described as from this source.

(3) In the male, seminal proteins from the genital tract and in particular from the prostate and bulbo-urethral glands, which open directly into the urethra. Several proteins have been described in seminal plasma (Ross, Moore, and Miller, 1942).

Immunochemical methods of analysis have made it possible not only to demonstrate such proteins in normal urine, but even to investigate their origins. The detection in normal urine of most of the plasma proteins (exceptions include fibrinogen and β-lipoprotein) was described previously (Grant, 1957): evidence for the presence of proteins from other sources is now presented.

Materials and Methods

Urines Examined.—Individual and pooled samples from four normal men, four normal women, and five children, three boys and two girls, aged 4 to 11, were investigated. They were all members of the laboratory staff or of their families. Simple precautions were taken to avoid contamination, and the samples were preserved with sodium azide (1 g. per litre). They were concentrated by ultrafiltration under negative pressure through Visking dialysis tubing as previously described, but, instead of tubing of $\frac{1}{2}$ in. flat width, longer lengths of the narrower $\frac{1}{4}$ in. flat width tubing† were used because of its ability to withstand the pressure without support (Everall and Wright, 1958). After the urine had been concentrated about 50 times, it was centrifuged and the supernatant was further concentrated in a fresh length of dialysis tubing to give a final concentration of about 1,000 times. If it is concentrated in a single stage, the final product is often too viscous to allow any precipitate to settle.

Tamm-Horsfall Mucoprotein.—This was prepared from normal urine (preserved with sodium azide, 1 g. per litre) by adding an equal volume of 1.16M NaCl. The precipitate was washed twice with 0.58M saline, and then dialysed against distilled water containing 1 g. per litre sodium azide, until tests for chloride became negative. Before analysis it was dialysed against the appropriate buffer.

Such preparations when freshly made will not diffuse through agar even in an electric field. On standing, however, they become in time freely diffusible and suitable for use in immunochemical agar gel tests.

Semen.—Samples were available from patients who were referred to the laboratory from the infertility clinic and subsequently found to be normal.

Antisera.—These were prepared in rabbits against (1) concentrated normal male urine colloids; (2) concentrated normal female urine colloids; (3) Tamm-Horsfall mucoprotein freshly prepared as above; and (4) normal human semen.

At least two rabbits were used for each; the courses of injection were as previously described; and the antisera were preserved with sodium azide (1 in 1,000). Before use antibodies against serum proteins were removed by absorption.

This absorption proved to be unexpectedly difficult, for the antisera were found to contain antibodies against proteins which are present in exceedingly low concentrations in normal human serum. Human serum, concentrated four times by the same technique as that used for concentrating urine colloids, was therefore used for their absorption, 1 volume of concentrate to 10 volumes of antiserum. The absorbed antisera then gave no precipitin reaction with an equal volume of human serum concentrate,

*Based on a paper read at the combined meeting of the Association of Clinical Pathologists and of the Association of Clinical Biochemists in London on October 4, 1958. Part I was read at a meeting of the Association of Clinical Pathologists at Torquay on October 12, 1957 (*J. clin. Path.*, 1957, **10**, 360).

†Obtainable from Hudes Merchandising Corporation, 52, Gloucester Place, London, W.1.

an equal volume of fresh serum, or 1/20 volume of fresh serum.

In addition to the above antisera, rabbit antisera against normal human serum proteins were required for the analyses of normal human serum run in parallel with the urine analyses to indicate the relative mobilities of the serum and urinary tract proteins.

Immunochemical Analyses.—For the simple agar gel precipitin tests (Ouchterlony, 1949) Petri dishes containing *p*H 7.4 phosphate agar were used (Gell, 1955). Then 27.5 g. potassium dihydrogen phosphate, 17.0 g. sodium chloride, 8.0 g. potassium hydroxide, and 15 g. agar were dissolved in 2 litres of distilled water and filtered while hot through a bed of "hyflo super cell" under reduced pressure. Sodium azide was added to give a final concentration of 1 g. per litre and the agar sterilized at 5 lb. pressure for 15 minutes.

The immuno-electrophoretic analyses were carried out by the method of Grabar and Williams (1955) modified as previously described (Grant, 1957).

Results

Analyses with Antisera against Normal Urine Colloids Absorbed with Normal Serum.—These antisera should demonstrate every urine protein except those in blood serum.

Simple Diffusion-precipitin Analyses.—Fig. 1 illustrates such an analysis; two samples of male urine concentrate, two samples of female urine concentrate, and some salt-precipitated Tamm-Horsfall mucoprotein have been allowed to react with an antiserum made against male urine colloids, prepared and absorbed as described above. The urine concentrates form multiple precipitin lines, two of which are particularly well marked. One of the latter gives the "reaction of identity" with the mucoprotein preparation. Similar results were obtained with the antisera made against female urine concentrates.

FIG. 1.—Comparison between non-serum proteins of male urine, female urine, and Tamm-Horsfall mucoprotein in peripheral cups. In the centre cup is shown antiserum against normal male urine colloids (absorbed with normal human serum). (Direct print of Ouchterlony plate stained with azocarmine.)

FIG. 2.—Comparison between male (M.U.), female urine colloids (F.U.), and semen (Se), using the same antiserum. (Similar direct print.)

In Fig. 2 urine concentrates have been compared with normal semen using a similar antiserum. As expected the antiserum contains antibodies to several seminal proteins and these proteins are only minor components of the urine protein; their precipitin lines cut those of the main urinary tract proteins, including that due to the Tamm-Horsfall mucoprotein, a protein evidently absent from semen.

Similar analyses, using also antisera made against female urine concentrates, led to a further and unexpected finding, namely, several trace components common to semen and to female urine as well as to male urine. On the basis of these preliminary experiments three groups of components can be distinguished:

(1) Components common to male and female urine but not present in semen: these presumably arise from the upper urinary tract; they include the mucoprotein of Tamm and Horsfall as well as the other principal components.

(2) Components common only to male urine and semen: they are apparently only present in traces and presumably arise from the genital tract.

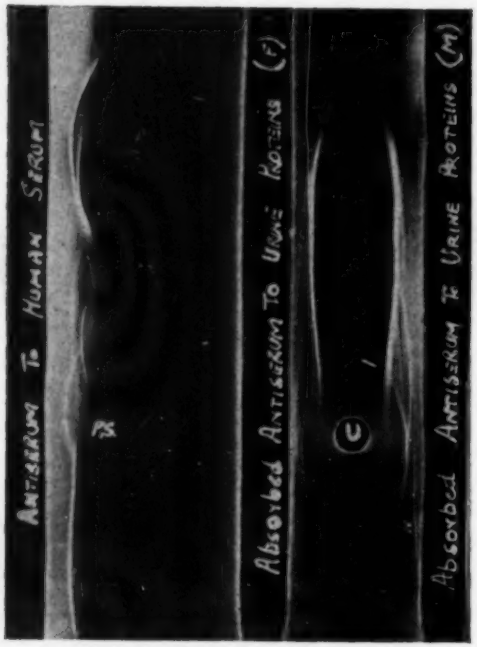

FIG. 3.—Immuno-electrophoretic analysis of male urine colloids (U.) with antisera against male non-serum urine proteins (M.) and female non-serum urine proteins (F.). On the left some normal pooled human serum (P.S.) has been analysed with its homologous antiserum for comparison. The additional line with the male antiserum is prostatic in origin. (Direct print after staining with azocarmine.)

(3) Components common to male urine, female urine, and semen: they seem to be present only in traces and possibly arise from the urethra; the female urethra has glands similar to, although less abundant than, those of the male urethra. (Normal cervical mucus appeared to give no reaction with the antisera.)

Immuno-electrophoretic Analyses.—Urine concentrates from either sex were again found to give very similar results whichever anti-urine-protein antiserum was used (Fig. 3). Such analyses suggest that there are at least a dozen antigenic components other than serum proteins in male urine, most of them giving only faint precipitin lines.

Analyses with Antisera against Normal Urine Colloids Absorbed with 1/10 Volume of Semen and with Serum.—These antisera should precipitate only the first group of components listed above, namely, those arising from the upper urinary tract (the kidneys, ureters, and bladder).

The two sexes gave a similar pattern, which includes the principal lines seen in the previous analyses. It was not significantly changed whether the absorbed antiserum used had been made against male or against female urine colloids. But, as commonly occurs in such immunochemical analyses, the results using different ratios of antigen (urine concentrates) to antiserum or using similar antisera from different rabbits, although fundamentally similar, differed in the relative densities of the precipitin lines. A composite diagram (Fig. 4) was therefore made from a number of such

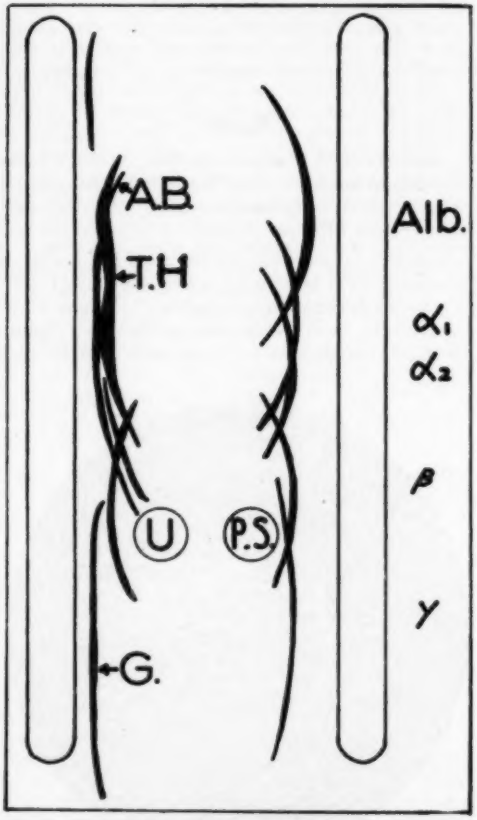

FIG. 4.—Diagram of immuno-electrophoretic analysis of upper urinary tract non-serum proteins (left) with outline of blood serum protein pattern (right) for comparison. U.=urine colloid concentrate. P.S.=pooled normal blood serum. Left trough=antiserum against male urine colloids, absorbed with serum and semen. Right trough=antiserum against normal human blood serum.

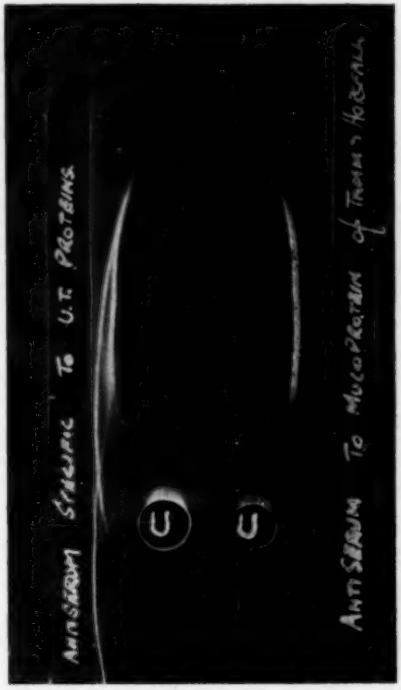

FIG. 5.—Identification of component antigenically similar to Tamm-Horsfall mucoprotein. U.=male urine concentrate. Left trough=antiserum against male urine colloids absorbed with serum and semen. Right trough=antiserum to the salt-precipitated protein of Tamm and Horsfall.

analyses as that illustrated on the left of Fig. 5. Comparison with serum proteins analysed on the same plate allows a rough estimate of the mobilities of these urine proteins using the data of Williams and Grabar (1955).

Of the six precipitin lines, that labelled T.H. in Fig. 4 was predominant in all analyses. The line AB and occasionally the line G were nearly as dense with certain antisera. The remaining lines were always faint.

The line T.H. corresponds to the salt-precipitated mucoprotein of Tamm and Horsfall (Fig. 5). It has two humps with maxima as shown and sometimes it extends faintly to a position opposite the cup, probably owing to adsorption to material remaining unmoved.

Analyses with Antisera against Normal Semen Absorbed with Normal Human Serum.—These antisera should react with all seminal proteins not common to blood serum. Proteins of this type in the urine presumably arise from the urethra or male genital tract: they form the second and third groups of components classified in the preliminary experiments above.

Simple Diffusion-precipitin Analyses.—In Fig. 6 the use of this type of antiserum to compare the antigenic components of male urine, female urine, and semen is illustrated. It confirms that there are about four components common to semen and to female urine as well as to male urine. The absence of the Tamm-Horsfall mucoprotein from semen was also confirmed.

FIG. 6.—The non-serum proteins common to urine and semen. Centre shows antiserum against normal semen (absorbed with normal human serum). M.U.=male urine colloid concentrate. F.U.=female urine colloid concentrate. Se=semen. P.S.=pooled normal serum control.

Immuno-electrophoretic Analyses.—As these components are only present in traces, the urine colloids were concentrated 4,000 times before analysis. It was then found that the two most intense precipitin lines common to both male and female urine had mobilities in the α β serum globulin range and in the γ serum globulin range respectively. In addition male urine has two denser lines of prostatic origin with α and β mobilities. Fig. 7 shows these lines diagrammatically; inconstant fainter lines have been omitted.

Analyses with Antisera against Normal Semen Absorbed with Female Urine Colloids and Blood Serum.—These antisera should react only with the second group of components, the specific proteins of semen, and not with those proteins which arise from the blood or the urinary tract.

In Fig. 8 a normal male urine concentrate has been compared with samples of normal semen, prostatic fluid, and seminal vesicle fluid, using an antiserum made against normal semen and then absorbed with one-tenth of its volume of normal female urine concentrate (× 1,000) as well as with serum. The female urine colloid concentrate used for absorption was pooled from six donors. The figure shows that there are two specific seminal proteins in this sample of male urine and that both are prostatic in origin.

Analyses with Antisera against Salt-precipitated (Tamm-Horsfall) Mucoprotein Absorbed with Normal Human Serum.—These antisera were used to identify the Tamm-Horsfall component in normal urine colloid concentrates as in Fig. 5, to compare it with the salt-precipitated preparations, and to study the cause of the elongation of their precipitin lines.

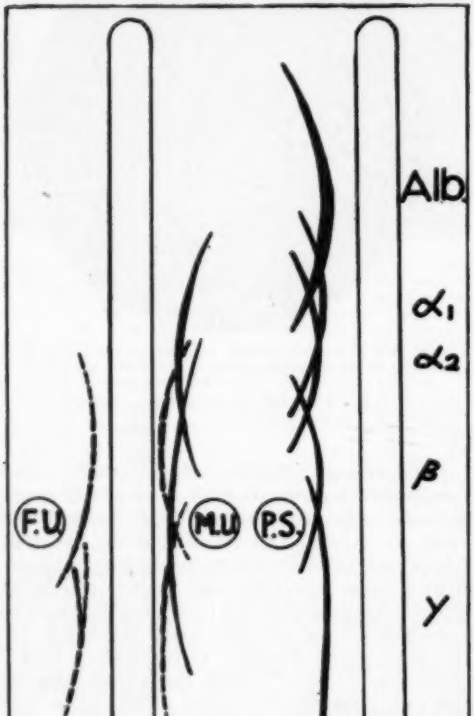

FIG. 7.—Diagram of immuno-electrophoretic analysis of lower urinary tract non-serum proteins (left) with outline of normal blood serum protein pattern (right) for comparison. M.U.=male urine colloid concentrate. F.U.=female urine colloid concentrate. P.S.=normal blood serum. Left trough=antiserum against normal semen absorbed with normal human serum. Right trough=antiserum against normal human blood serum.

FIG. 8.—Specific seminal proteins in male urine. Centre shows antiserum against normal semen absorbed with pooled female urine concentrates as well as normal serum. Se=semen. P.F.=prostatic fluid (collected post mortem from the outer cut surfaces of the lateral lobes). S.V.F.=seminal vesicle fluid (collected post mortem).

The diffusible form of the salt-precipitated mucoprotein was found to resemble the antigenically similar component of urine colloid concentrates in having the mobility of an α serum globulin, that is to say, a mobility much slower than the preparations studied by Tamm and Horsfall after freezing and drying. The line was also elongated but without the humps.

The elongated shape of the precipitin lines seen in Figs. 3 and 5 suggests that in normal urine this mucoprotein is not a single protein, but, like γ globulin, a mixture of closely related ones, identical antigenically but differing in mobilities. The two humps suggest that there are two main forms. The possibility that the appearance is due to adsorption on to α serum globulins seems ruled out by the low concentrations of these globulins in urine (Grant, 1957). Adsorption on to the agar was excluded by two-dimensional electrophoresis (Fig. 9). A drop of concentrated urine colloids was placed in a hole near one corner of a rectangular plate of barbiturate agar and submitted to electrophoresis in the usual way. The plate was then turned through a right angle and submitted to electrophoresis a second time so that the proteins moved up between troughs previously cut

FIG. 9.—Demonstration of heterogeneity of Tamm-Horsfall protein in normal urine colloid concentrate (see text).

in the plate. Finally the troughs were filled with an antiserum made specifically against the mucoprotein. It will be seen that the fractions with the greatest mobilities in the first run also have the greatest mobilities in the second run. If the protein had been homogeneous and adsorbed on to the agar, all the precipitin curves should have been similar.

Discussion

The analyses described above suggest that normal urine contains, in addition to plasma proteins, many antigenic components which are not detectable in blood and which arise from different parts of the urinary tract. Furthermore, urine is so complex as to make it probable that other components not described above would be detected, if an even more sensitive technique were used. This multiplicity of components may well explain the difficulties which have been met in the attempts to explain the relation, if any, between urinary calculus formation and the excretion of protein by the urinary tract.

If these urinary tract proteins occur also in blood, they must be in exceedingly small concentration. The failure to detect them immunochemically indicates that their concentrations must be less than about 1 mg. per 100 ml. Furthermore, rabbits repeatedly injected with blood serum over many months did not make detectable amounts of antibodies to them; such antisera after being absorbed with one-tenth volume of normal pooled serum failed to react with male or female urine concentrates on an Ouchterlony plate.

Owing, however, to the high normal glomerular filtration rate (180 litres per day), very small concentrations could give easily detectable amounts in urine concentrated a thousandfold, if the components were completely excreted by the kidney and not almost entirely retained like ordinary plasma proteins: their concentrations in the urine colloid concentrates could be some 100,000 times that in the blood. In view of this the possibility of the presence of these components in minute amounts in the blood cannot be regarded as wholly excluded.

Normal urine colloids are known to include free as well as protein-attached polysaccharides (Rigas and Heller, 1951; Kerby, 1954; Hamerman, Hatch, Reife, and Bartz, 1955; Markham, Jacobs, and Fletcher, 1956; Heremans, 1958). This can be illustrated by submitting a urine colloid concentrate to paper electrophoresis and staining the paper with Alcian blue for polysaccharide followed by azocarmine for protein: at least two bands due to free acid polysaccharides can be demonstrated migrating in front of the albumin (Heremans, Vaerman, and Heremans, 1959). These free polysaccharides do not, however, appear to be antigenic, for no precipitin lines were obtained when the appropriate areas of filter paper were tested with the antisera. Perhaps all the antigenic components are proteins, some no doubt having large polysaccharide radicals attached; the possibility, however, remains that normal urine contains some free polysaccharides sufficiently complex to be antigenic.

Tamm and Horsfall (1952) found normal urine to contain about 25 mg. per litre of their mucoprotein, so forming a considerable proportion of the traces of protein known to be present. It was not therefore surprising to find that the most prominent non-serum protein precipitin line should give the "reaction of identity" with this salt-precipitated mucoprotein.

But, as has been shown, the shape of this line and the results of two-dimensional electrophoresis indicate that there are at least two forms of this protein in normal urine. If several interchangeable forms differing in molecular size can exist, it would help to explain why each published method for the isolation of the mucoproteins of normal urine seems to result in a product with different properties. Tamm and Horsfall (1952) used salt precipitation and isolated a mucoprotein with thread-like molecules of molecular weight 7 million having an electrophoretic mobility much

FIG. 10.—Comparison between male urine colloid concentrate (M.U.) and mucoprotein preparations precipitated with sodium chloride (T-H), cetyl trimethyl ammonium bromide (cetab), and benzoic acid (B_1 and B_2). Centre shows antiserum to male urine colloids (absorbed with human blood serum).

greater than that of albumin. Boyce, Garvey, and Norfleet (1954) describe the mucoprotein in concentrated normal urine colloids as partly insoluble, their "uromucoid," and partly soluble with the mobility of an α serum globulin, in agreement with the findings above. Anderson and Maclagan (1955) isolated their mucoprotein by precipitation with benzoic acid and found it to have the electrophoretic mobility of an α serum globulin and a molecular weight of about 30,000 (see Maclagan and Anderson, 1958). Di Ferrante and Rich (1956) used cetyl trimethyl ammonium bromide as precipitant. Their product resembled Tamm and Horsfall's product in its biological activity and chemical analysis (Di Ferrante and Popenoe, 1957), but was found by Maxfield (1958) to have a molecular weight of 1.5 million.

Presumably all these forms have a precursor in common. Their antigenic similarity (see Fig. 10) is good evidence that this is so, as well as the fact that our fresh preparations of the salt-precipitated protein, when diffusible, have α serum globulin mobility like the corresponding component in concentrated urine colloids; they also have the same ultra-violet adsorption spectrum (maximum 277 mμ) as the preparations of Tamm and Horsfall and of Di Ferrante and Rich.

The physical differences between the different preparations are presumably due to varying degrees of polymerization, but it is not clear which is the naturally secreted form, a small molecular form which polymerizes during isolation, or a large molecular form which breaks down. Another possibility is that the antigenic components with α serum globulin mobility are protein moieties of an originally more complex mucoprotein or mucopolysaccharide.

The heterogeneity of this mucoprotein fraction is evidently the main reason why electrophoresis of concentrated normal urine colloids produces a blurred pattern in the α serum globulin region, both by the moving boundary (Rigas and Heller, 1951) and by the paper (Slater and Kunkel, 1953) methods.

The precipitin lines of some of the minor urinary tract components also seem elongated and asymmetrical. This may be due to similar heterogeneity, or to absorption either to agar or to other proteins.

Summary

About 12 antigenic components, not detectable in normal serum, have been demonstrated immunochemically in normal urine.

These components, probably all proteins, are divisible into three groups:

(1) Those arising from the kidneys, ureters, and bladder. There are about six of these and they include all the principal non-serum proteins in normal urine.

(2) In the male, trace components from the genital tract, especially from the prostate.

(3) Trace components common to male urine, female urine, and semen, possibly arising in both sexes from the urethra.

The mobilities of the more prominent components have been studied by immunoelectrophoresis and compared with those of the principal serum proteins.

Included in the first group is the main non-serum protein of normal urine. It gives the "reaction of identity" with the main component of the proteins precipitated by sodium chloride (Tamm and Horsfall), benzoic acid (Anderson and Maclagan), or cetyl trimethyl ammonium bromide (Di Ferrante and Rich). The differences which do occur between these various mucoprotein preparations are probably due to varying degrees of polymerization.

This mucoprotein and possibly some of the other non-serum proteins are present in urine as groups of closely related proteins rather than as single substances.

We should like to thank Mr. G. Wright and Miss M. Carpenter for technical assistance and Mr. R. Ross for Figs. 4 and 7.

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A COMPARISON OF METHODS FOR THE ESTIMATION OF SERUM CHOLESTEROL AND VALUES IN RANDOM SAMPLES OF POPULATIONS IN THE 55-64 AGE GROUP

BY

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The increasing mortality from coronary heart disease has focused attention on its epidemiological and biological aspects, and in the latter connexion much work has been done on the relationship between blood cholesterol levels and heart disease. As a result many methods for the estimation of cholesterol levels in serum have been published and their multiplicity indicates the difficulties of the estimation. Reviews published by Zak and Ressler (1955) and Cook (1958) deal with the isolation and estimation, but few comparisons of more than two methods are available.

The estimation has usually to be done on small amounts of material so that semi-micro colorimetric methods are normally used. The majority of these are based upon the Liebermann–Burchard reaction. Liebermann described the colour changes which occur when concentrated sulphuric acid is added to a solution of cholesterol in acetic anhydride, and Burchard later developed this by applying the reaction in a non-aqueous solvent such as chloroform. The more sensitive method of Zlatkis, Zak, and Boyle (1953) makes use of the colour developed by cholesterol with concentrated sulphuric acid and ferric chloride. Several modifications of this method have been published, which suggests that it has some technical difficulties.

The Methods

This paper gives the results of an experiment comparing several methods in current use for the determination of serum cholesterol. The methods chosen were as follows:

(1) The method of Pearson, Stern, and McGavack (1953) was used as published. In it, *p*-toluenesulphonic acid and acetic anhydride are added to the serum, followed later by concentrated sulphuric acid. The colour develops rapidly.

(2) The method of Zlatkis *et al.* (1953) was also used as published. In it, a mixture of glacial acetic acid, ferric chloride, and concentrated sulphuric acid is added to the serum and the colour measured after a short interval. The results obtained by this method, although consistent among themselves, were very much higher than all the others so that a further investigation was done to examine the effect of purifying the acetic acid used, by the method of Henly (1957).

(3) In this, the method of Trinder (1952), the cholesterol esters in the serum are decomposed by a treatment with alcoholic potash and then all the cholesterol in solution is extracted with petroleum ether. After evaporation of this solvent the cholesterol is dissolved in 1 : 2 dichloroethane and a colour developed by the addition of a mixture of acetyl chloride and concentrated sulphuric acid.

In the original method as published the colour was measured on an absorptiometer with a filter giving a fairly wide waveband of light. The instrument used in the trial was a "unicam" S.P. 600 in which the wavelength of light can be chosen more accurately. Preliminary work showed that the maximum absorption of light occurred at 490 mμ, so this wavelength was used throughout.

(4) Sackett's (1925) method is a modification of Bloor's (1916) method. The serum is treated with a mixture of alcohol and ether to precipitate proteins and extract the cholesterol. The dissolved cholesterol is recovered by evaporation and then re-dissolved in chloroform to which is added a mixture of acetic anhydride and concentrated sulphuric acid to develop a colour.

The method used here was essentially the same except that time and temperature were standardized, since these factors are critical. Preliminary trials showed that at 25° C. the colour developed rapidly to a maximum and faded almost as rapidly, as is shown in Fig. 1. Strict time-keeping is therefore necessary and it is essential to time all operations so that readings are taken exactly 15 minutes after the addition of the colour reagent. The effect of varying

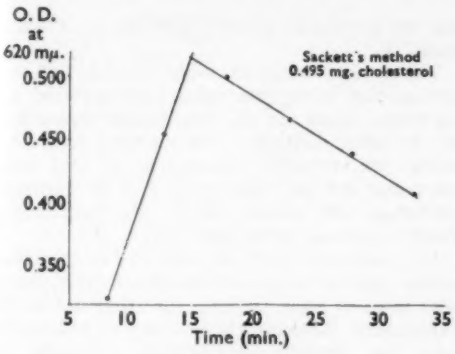

FIG. 1

temperatures was not investigated closely, but 25° C. was chosen arbitrarily, since it is high enough to be above laboratory temperature and therefore a thermostat can be used, and low enough to make cooling unnecessary before measuring the colour.

(5) The method of Abell, Levy, Brodie, and Kendall (1952), as modified by Anderson and Keys (1956), is the K.5 modification which has been used by Keys on his surveys. The serum is treated with alcoholic potash and the cholesterol then extracted with petroleum ether. After evaporation, the dry residue of cholesterol is dissolved in a mixture of acetic anhydride, sulphuric acid, and acetic acid to develop the colour. To overcome the fading difficulties, readings of the colour are taken at intervals of time and the maximum colour developed thus obtained by extrapolation.

(6) The method of Schoenheimer and Sperry (1934), as modified by Sperry and Webb (1950), was used. In this the serum is treated with alcohol and acetone and after filtration of the protein floc the cholesterol in the filtrate is precipitated as the digitonide by the addition of digitonin solution.

The precipitate is separated, dissolved in hot glacial acetic acid, and then the colour developed by the addition of an acetic-anhydride-sulphuric acid mixture. Strict time-keeping is again necessary, but only one reading of the colour is taken.

Test Samples

The sera used were chosen on the basis of preliminary tests so that the cholesterol content was spread over as wide a range as possible. Since it was impossible to do all the determinations in one day, the samples had to be stored. It is generally assumed that when serum is frozen the esterified cholesterol level may change but the total cholesterol level remains unaffected, although little experimental evidence seems to be available. A method of determination of total cholesterol which does not decompose esters may therefore give different results in a fresh serum from those on the same serum after freezing. Storing at 4° C. does not affect the esterified cholesterol. Two methods of storage were used in this test. In the first enough of the sample for a determination in triplicate, by any one method, was placed in a bijou bottle and kept in deep freeze for at least 30 days to stabilize any changes due to freezing. In the other method of storage a bulk sample of each serum was kept in the refrigerator at 4° C. and aliquots measured out as required.

Standard Cholesterol Solution

The results given by all the methods are based upon a solution of cholesterol of known strength using cholesterol of known purity, which in these tests was B.D.H. cholesterol recrystallized from absolute alcohol and dried to constant weight over phosphorus pentoxide in a partial vacuum. The melting point of the final product was 148.5–149.5° C. Aliquots of this solution were pipetted out and evaporated to dryness in suitable tubes. The solid residue of cholesterol was then dissolved in measured amounts of whichever solvent the particular method in use required.

Samples from Randomly Selected Populations

Groups of males and females between the ages of 55 and 64 selected at random from the population in the Vale of Glamorgan have been studied by Thomas, Cochrane, and Higgins (1958) and in the course of this study blood samples were taken. The samples of blood were drawn from the arm vein and no attempt was made to take the samples at definite intervals after meals. The patients were invited to attend the clinic and whenever they arrived the sample was drawn, so that it could have been taken at any time from 9 a.m. to 9 p.m. After sampling, the blood was allowed to clot naturally, ringed, and kept in a refrigerator overnight, transported to the laboratory, and the serum separated. Blood samples were also obtained under similar conditions from males of the same age group in a survey in Annandale carried out by Kennedy and Cowan (1958). In both surveys over 90% of the patients in the sample chosen were actually examined.

Results

The results obtained by the different methods on the samples used are given in Table 1. Unfortunately the results by the method of Pearson *et al.* on Samples 1 to 5 were lost.

A statistical analysis has been done on the results after the figures obtained by the methods of Pearson *et al.* and Zlatkis *et al.* have been excluded since these were obviously much higher than any of the others.

The standard error of a single determination by Trinder's, Keys and others' (K.5), and Sperry and Webb's methods did not differ significantly, but the error of Sackett's method is significantly higher. The mean value obtained by Sackett's method is significantly higher than that of the

TABLE I

CHOLESTEROL LEVELS (MG. %) IN SERUM OBTAINED BY DIFFERENT METHODS

		Methods					
	Sample	Pearson *et al.* (1953)	Zlatkis *et al.* (1953)	Trinder (1952)	Sackett (1925)	Anderson and Keys (K.5) (1956)	Sperry and Webb (1950)
Stored in deep freeze	1	—	240	126	132	121	125
			246	126	137	112	125
			246	122	150	115	126
	2	—	300	179	173	169	168
			299	177	176	168	169
			293	176	188	167	169
	3	—	330	224	233	222	224
			328	225	231	220	228
			327	222	213	213	225
	4	—	391	303	299	286	296
			397	299	316	288	296
			397	299	300	288	291
	5	—	446	344	352	337	327
			426	338	371	329	329
			446	342	360	336	314
Mean ..		—	340·8	233·4	242·1	224·7	227·5
Estimated standard error of a single determination		—	5·90	2·22	9·52	3·60	3·99
Stored in refrigerator	6	154	252	122	121	110	117
		164	251	120	121	114	121
		155	247	122	130	111	117
	7	190	263	161	151	146	162
		191	254	159	148	156	165
		193	259	—	153	152	163
	8	226	298	206	225	201	206
		225	315	210	226	201	203
		230	309	209	193	197	204
	9	366	444	347	385	340	328
		372	432	339	376	336	329
		377	419	348	380	343	322
	10	209	284	195	200	186	189
		208	275	194	198	184	187
		211	272	189	204	193	185
Mean ..		231·4	304·9	208·6	214·1	198·0	199·9
Estimated standard error of a single determination		3·80	7·71	2·87	9·11	3·73	2·38
Mean of all samples		231·4	322·9	221·5	228·1	211·4	213·7
Estimated standard error of a single determination		3·80	6·86	2·64	9·32	3·67	3·29

other three methods, and the mean by Trinder's method is significantly higher than that of Anderson and Keys (K.5) and Sperry and Webb's methods, which agree.

As mentioned earlier, the high results obtained by the method of Zlatkis *et al.* prompted a further study of the effect of purifying the acetic acid, which entails refluxing the acetic acid with chromium trioxide. Results with treated acid and the same acid before treatment are given in Table II.

The results obtained with treated acid are considerably lower than those with untreated, but the mean values are still significantly higher than all the other methods. The standard error of a single determination was reduced by acid treatment and did not differ from that of Trinder's, Anderson and Keys's (K.5), and Sperry and Webb's methods found previously.

It is apparent from the current literature that results obtained by this method usually agree better with those of other methods than they did in this experiment. This experience seems to indicate the need for considerable caution in using the technique as published.

Random Sample of Population

Cholesterol levels were determined by Trinder's method and the results are given in Figs. 2 and 3.

Discussion

Results of Analyses by Different Methods.—The methods chosen can be divided into two types on the basis of technique. In the methods of Pearson *et al.* and Zlatkis *et al.* the reagents are added directly to the serum and in the others the cholesterol is extracted, before estimation, either

TABLE II

CHOLESTEROL LEVELS (MG. %) IN SERUM ESTIMATED BY METHOD OF ZLATKIS *ET AL.* (1953)

Sample	Untreated Acid	Treated Acid
1	272	161
	281	162
	276	161
2	336	219
	324	215
	338	215
3	380	270
	374	270
	367	272
4	446	347
	457	351
	457	356
5*	—	—
6	305	169
	302	170
	308	171
7	296	208
	307	208
	302	208
8	358	267
	360	266
	358	266
9	531	411
	526	403
	531	406
10	380	266
	365	273
	361	270
Mean	366·6	257·8
Estimated S.E. of single observation	5·86	1·96

* There was no sample available for a repeat determination.

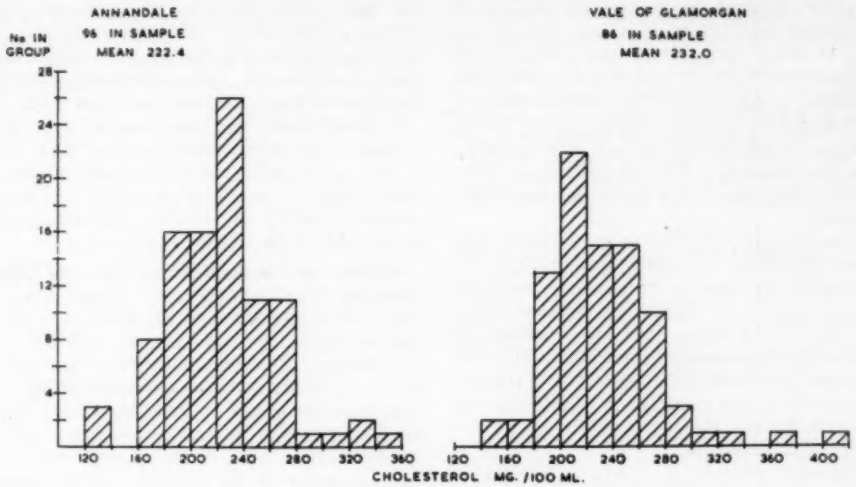

FIG. 2.—Total serum cholesterol in males aged 55–64 years.

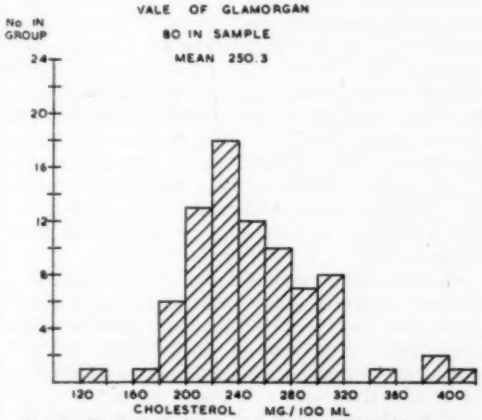

FIG. 3.—Total serum cholesterol in females aged 55–64 years.

by solvents or by digitonin precipitation. The results show that the methods in which the reagents are added to the serum are less accurate than the others. Since all sera are coloured, some strongly, the use of these methods necessitates the setting up of blanks for each sample and this cuts down the number of samples that can be done. It is not clear, also, that other substances in the serum are inert to the colour reagents, and in fact a recent modification of the method of Zlatkis *et al.* does extract the cholesterol from the serum (Herrmann, 1957). In this method the sulphuric acid-ferric chloride reagent is very viscous and the final mixture of glacial acetic acid and the reagent is also viscous, which means that transferring the solution from the tubes to colorimeter cells is difficult if air bubbles are to be avoided. Also washing the cells out after measuring the colour is tedious. This latter is, of course, a small point, but in a series of determinations using a time-table such small points can assume importance.

In the methods of Sackett, of Trinder, and of Anderson and Keys the cholesterol is extracted by organic solvents and in the Sperry and Webb method by precipitation with digitonin. Of the organic solvent methods, in that of Sackett a mixed alcohol-ethyl ether solvent is employed and, in the other two, petroleum ether. Sackett's method is the least satisfactory in that two transfers of the cholesterol are required, which doubles the chance of loss, and also a time-consuming centrifugation is necessary.

In the petroleum ether methods, the extraction follows a treatment with alcoholic potash designed to saponify any esters of cholesterol. The method of Anderson and Keys (K.5) employs two separate extractions while that of Trinder one only. From the results it appears that one is quite as efficient as two, since the values by Trinder's method are the same as those with Anderson and Keys's.

The colour is developed in the methods of both Sackett and Anderson and Keys by a sulphuric-acid-acetic-anhydride mixture. It has to be prepared freshly each day, as do all the colour reagents in the methods used, but the conditions of preparation involve cooling in an ice-bath, which is not always convenient.

In Trinder's method the reagent is a mixture of acetyl chloride and sulphuric acid. It can be prepared at room temperature and used at that temperature. It reacts violently with water, but under normal conditions of the method there is no water present. It fumes slightly in air and it is convenient to add the reagent in a fume chamber but it is not essential. The reagent is no more unpleasant than the acetic-anhydride-sulphuric-acid reagent.

The green colour in the methods of Sackett and Anderson and Keys is labile, and in Sackett's method much care has to be taken to read the intensity of colour at exactly 15 minutes after addition of the reagent. In the method of Anderson and Keys readings are taken at intervals and the peak intensity determined. In Trinder's method the red colour develops in 15 minutes and is then stable for at least an hour although careful time-keeping is advisable.

The digitonin method of Sperry and Webb is not usually used for routine work, since it involves an overnight wait for precipitation of the digitonide. The pre-treatment using alcohol-acetone is liable to give trouble in that the mixture on the steam bath can "bump" badly with loss even from narrow-necked flasks.

When considering methods in the foregoing manner it is very difficult to avoid bias. In this laboratory the Trinder method has been adopted and many hundreds of determinations have been done. As a result, other methods tend to appear tedious and awkward. This may be due to real difficulties in the techniques or it may be due to the fact that, for the small amount of work necessary for the comparison, the techniques have not been used enough to become familiar and therefore easier.

It is of interest here that in the Johns Hopkins School of Medicine, Thomas and Eisenberg (1957), using an adapted Sackett method, obtained standard errors on separate occasions of 11.2 and 10.1 mg. cholesterol per 100 ml., which are similar to the 9.3 obtained here. They also quote figures by other workers using the method of Abell *et al.* of 7.6 to 14.5 mg. per 100 ml., depending on the workers. Anderson and Keys's modification of the method of Abell *et al.* here gave a standard error of 3.7 mg. per 100 ml., which is considerably better.

It would appear, however, that the best methods for use on a routine basis are either those of Anderson and Keys or Trinder, and the latter is quicker, since serial readings of colour are not taken. From considerable experience of the method and comparisons of results obtained by workers using the (K.5) modification of Anderson and Keys with those on the same material by Trinder's method, the difference was thought to be less than the 4% found here. In Trinder's original paper his own comparisons with the Sperry and Webb method are closer than 2%.

It is not possible to compare the two methods of storage on the cholesterol level since samples of the same sera were not kept under both conditions. The standard error of a single determination by any method does not appear to be affected by the type of storage.

Results of Determinations on the Random Samples.—The populations from which the random samples were drawn were, in both cases, predominantly agricultural, although there was, of course, a proportion of professional and industrial workers and some retired persons. The particular age group of 55–64 does not appear to have been studied as such to any great extent, but several workers have studied populations with age groups contained in, or containing, this group.

Adlersberg, Schaefer, Steinberg, and Chun-I Wang (1956), using the Schoenheimer digitonin method, examined a group of males and females, chiefly of Italian, Irish, English, and Scottish origin, in New York, and in the age group 58–62 found a mean cholesterol level among the males of 236.2 and females of 263.8 mg. per 100 ml. In the present survey in this age group the mean cholesterol level in the Vale of Glamorgan was 240.2 for the males and 259.1 mg. per 100 ml. in the females, while in Annandale the level for males was 223.9 mg. per 100 ml., which is quite good agreement.

Epstein, Boas, and Simpson (1957) studied a group composed almost entirely of Italians and Jews, so that their results are probably less comparable than those of Adlersberg *et al.* with the present series, but they did find that, regardless of origin, the mean cholesterol level (using the method of Abell *et al.*) of the females was higher than that of the males. In the present study the mean cholesterol level of the males of both groups did not differ significantly, but that of the females in the Vale was significantly higher.

Within the age group studied, although the numbers are small, a breakdown into separate years showed a general upward trend in mean cholesterol level over the years 55–59 and then a fall to the age of 64, which appears to be fairly consistent with the findings of both Adlersberg *et al.* and Epstein *et al.*

Summary

Increasing mortality from coronary disease has focused attention on the cholesterol level of blood

as one of the many factors connected with the aetiology of the disease.

Many methods have been proposed for the difficult estimation of blood cholesterol levels, and six of these methods, namely, those of Pearson *et al.* (1953), Zlatkis *et al.* (1953), Trinder (1952), Sackett (1925), Anderson and Keys (K.5) (1956), and Sperry and Webb (1950), have been compared by carrying out determinations in triplicate by each method on 10 sera.

The method of Trinder was the most reproducible, with those of Anderson and Keys (K.5) and Sperry and Webb the next most reproducible. Sackett's method was the least reproducible.

Mean levels obtained with the methods of Sperry and Webb and Anderson and Keys (K.5) did not differ ; the mean by Trinder's method was 4.2% higher and the mean by Sackett's method 7.3% higher than these two. The methods of Pearson *et al.* and Zlatkis *et al.* gave means 8.8% and 51.8% higher respectively.

The methods of Trinder and Anderson and Keys (K.5) are the most suitable for routine use, and Trinder's method involves less manipulation and is quicker.

Cholesterol levels in random samples of populations in different areas, in the age group 55–64, were determined by Trinder's method to gain basic information for comparative purposes.

The author wishes to place on record his thanks to Dr. J. C. Gilson for his help and encouragement and to Drs. A. L. Cochrane and A. C. Kennedy for their help in organizing the samples. Especial thanks are due to Mr. P. D. Oldham for his keen interest and for his invaluable assistance in planning the experiment and to Mr. Peter Cross for analysing the results.

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ORGANIC ACID EXCRETION AFTER CALCIUM GLUCONATE INFUSIONS

BY

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Infusions of calcium gluconate are now often used in the investigation of skeletal disease. Nordin and Fraser (1956) found that osteomalacic patients retained an abnormally large proportion of the infused calcium in bone, with a corresponding reduced excretion of calcium in the urine. The hypercalcaemia after the infusion causes a fall in urinary phosphate clearance and rate of phosphate excretion thought to be due to a temporary reduced output of endogenous parathyroid hormone (Nordin and Fraser, 1954). Calcium infusions also cause diuresis with an increased urinary loss of sodium, potassium, and chloride (Levitt, Halpern, Polimeros, Sweet, and Gribetz, 1958; Freedman, Moulton, and Spencer, 1958). Freedman *et al.* (1958) report "a threefold rise in excretion of undetermined anion which was possibly due to an increase in citrate excretion." The observations reported in this paper were designed to investigate this "undetermined anion" in more detail. It is shown that after calcium gluconate infusions the level of urinary organic acid rises appreciably, and is, as might be expected, chiefly gluconate. Urinary citrate is usually somewhat increased, but evidence is given that this is secondary to the rise of urinary calcium.

Methods

The infusions of calcium gluconate were carried out in the morning between 8 a.m. and 1 p.m. A basal specimen of urine was collected in the 12 hours immediately preceding the infusion. One hundred millilitres of 10% calcium gluconate added to 500 ml. of 5% glucose solution were infused at a steady rate over a five-hour period. Separate timed urine specimens were collected for the next 24 hours. No attempt was made to obtain urine samples at fixed times, as the infusions cause a variable and unpredictable diuresis, and it was desired to avoid collection errors as far as possible. All the samples were analysed for calcium, citrate, and total organic acid. The specimens containing most organic acid were also analysed for keto-acids, and the organic acids were examined qualitatively by paper chromatography.

The results are reported of 10 infusions classified as follows: (1) five infusions in healthy adult subjects in normal acid-base balance; (2) two infusions in healthy adult subjects made mildly acidotic by the ingestion of ammonium chloride, 2 g. four times daily for the two days preceding the infusion and the day of the infusion; (3) two infusions in adults suffering from severe osteomalacia due to idiopathic steatorrhoea; and (4) one infusion in one of the osteomalacic patients after four weeks' treatment with large doses of "calciferol" (1 m. units daily), resulting in considerable biochemical and radiological improvement.

Chemical Methods

Urinary Calcium.—The method of Shohl and Pedley (1922) was used.

Urinary Titratable Organic Acid.—A modification by Palmer (1926) of the original titration method of Van Slyke and Palmer (1920) was chosen.

Urinary Citrate.—The method of McArdle (1955) was used.

Urinary Keto-acids.—The method of McArdle (1957) was used.

Chromatography of Urinary Organic Acids.—Aliquots of the control and experimental urines were evaporated to dryness under reduced pressure. The residue was dissolved in a 1:1 mixture of methyl alcohol and water and chromatographed on Whatman No. 1 paper using three different solvent systems (one-way chromatography), namely, (1) butanol-acetic acid-water (120:30:50), (2) methanol-ethanol-N ammonia (45:45:10), (3) isopropanol-pyridine-water (120:40:40). The location reagents were silver nitrate and lead tetra-acetate.

Results

The changes produced in the excretion of calcium, citrate, and organic acid are shown in Figs. 1–3. Fig. 1 gives the effect on calcium output. There was no significant difference in calcium excretion in the normal and acidotic subjects. An average of 45% (range 39%–52%) of the infused calcium appeared in the urine. Calcium excretion was highest just after the end

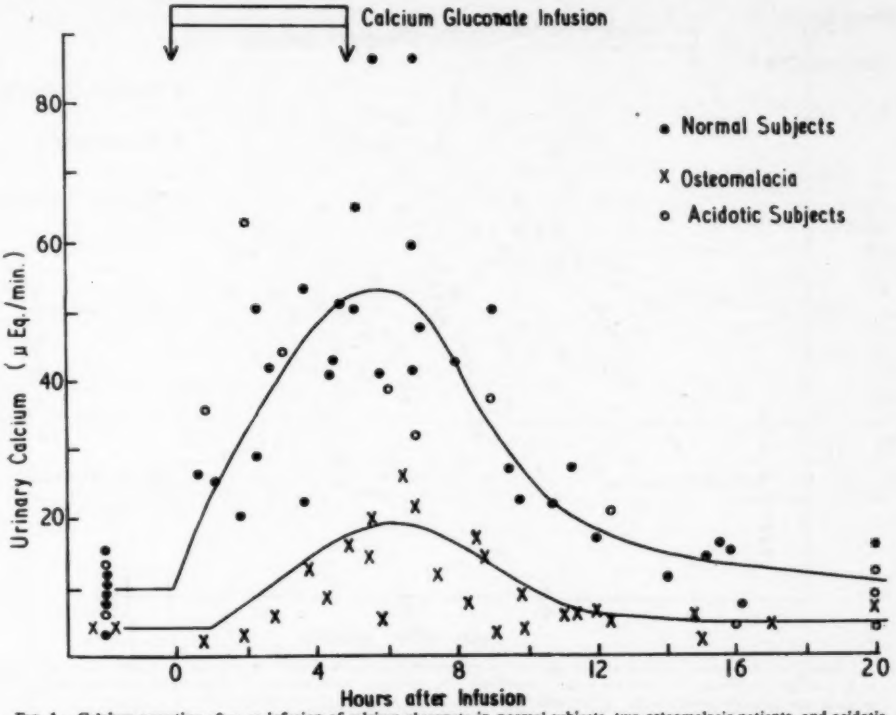


FIG. 1.—Calcium excretion after an infusion of calcium gluconate in normal subjects, two osteomalacic patients, and acidotic normal subjects. Calcium excretion is unaffected by acidosis but is greatly reduced in the osteomalacic patients. The continuous lines give the mean excretion in normal and acidotic subjects and in the osteomalacic patients respectively.

of the infusion and had returned to basal levels within 20 hours. Both the basal calcium excretion and the amount excreted after the infusion were abnormally low in the osteomalacic patients, a mean of 10% (range 8%–12%) of the injected calcium being excreted. These values agree with the more extensive observations of Nordin and Fraser (1956), who reported a range of 33% to 53% calcium excreted in normal subjects, and less than 27% in cases of osteomalacia. To simplify the figure, the results obtained in the osteomalacic patient after treatment are not plotted in Fig. 1. The basal value of urinary calcium had not increased, but excretion of infused calcium rose from 8% to 22%.

Changes in total urinary organic acid are given in Fig. 2. The basal excretion varied from 20 to 65 μEq./min., which is a larger range than that reported in normal subjects by Van Slyke and Palmer (1920) of 28 to 52 μEq./min. The lowest values were in the acidotic subjects, in whom citric acid and keto-acid excretion is usually reduced (Evans, MacIntyre, Macpherson, and Milne, 1957). After the infusion, urinary organic acid levels rose in all subjects, there being no significant difference between the four groups. Excretion was maximal immediately after the end of the infusion and had fallen to basal levels within 20 hours. The chromatographic separation showed that there was a slight increase of citric acid, but the main change was the appearance of a large spot with R_f values identical with those of pure gluconic acid (R_f values 0.03, 0.67, and 0.31 in systems 1, 2, and 3). The infusion did not affect the excretion of either α-ketoglutaric acid or pyruvic acid. If it is assumed that gluconate excretion can be estimated by the increase in total urinary organic acid less the rise in urinary citrate, the average urinary excretion of infused gluconate was 72% (range 64%–82%). Stetten and Topper (1953) found that after injection of gluconate labelled with ^{14}C in rats, 60%–85% was excreted as gluconate in the urine, the remainder being metabolized.

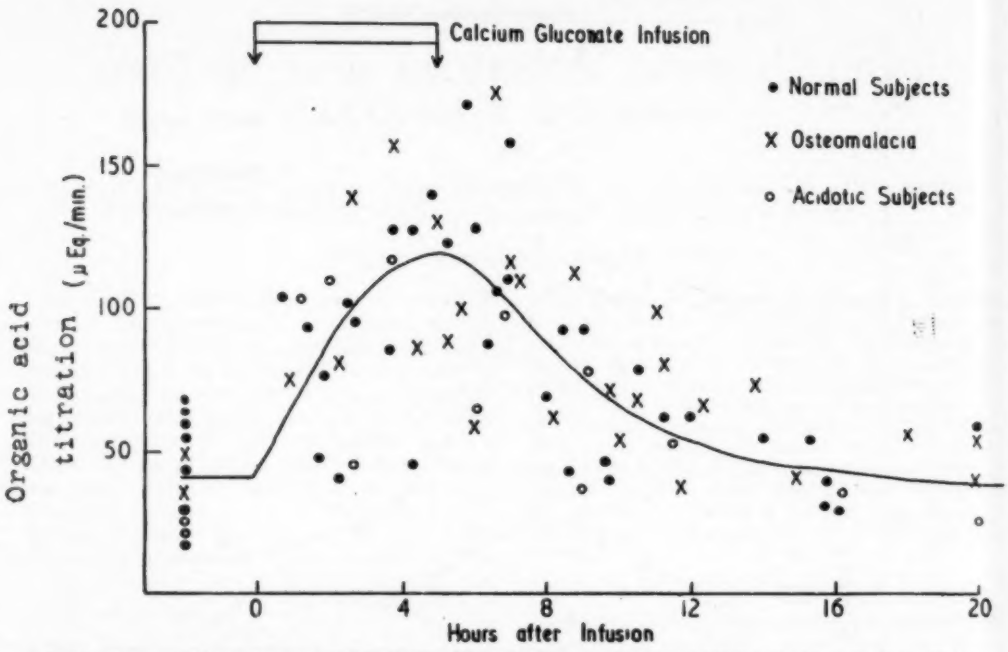


FIG. 2.—Excretion of titratable organic acid by the Van Slyke and Palmer method after an infusion of calcium gluconate. There is a considerable increase of urinary organic acid after the infusion with no significant difference in normal subjects, acidotic subjects, and cases of osteomalacia. The continuous line gives the mean of all observations.

Figs. 3 and 4 show the effect of the infusion on citrate excretion. The basal excretion varied from 0.8 to 1.8 μmol./min. in the normal subjects, but was less both in the acidotic subjects and in the untreated cases of osteomalacia. Östberg (1931) gave the normal range of citrate excretion in adults as 0.7 to 3.6 μmol./min. In the treated case of osteomalacia, the basal urinary citrate output had risen to 2.5 μmol./min. Calcium gluconate infusion had no effect on citrate excretion in the acidotic subjects and in the untreated cases of osteomalacia. In the normal subjects, citrate output increased in parallel with urinary calcium (Fig. 4), the maximum observed output being 5.0 μmol./min., which is slightly above the normal range. In the treated case of osteomalacia, there was a similar increase in citrate to a maximum excretion rate of 5.9 μmol./min. Fig. 4 shows that in the normal subjects there was a positive correlation between urinary citrate and calcium ($r = +0.81$; $P < 0.001$). The mean increase of urinary citrate was 1 μmol./min. for every 20 μEq./min. rise in urinary calcium. Since the formula of the calcium citrate chelation complex (Hastings, McLean, Eichelberger, Hall, and Da Costa, 1934) is $(CaCit)^-$, this is only one-tenth the expected rise if all the excess calcium were excreted as chelate.

Discussion

Infusions of calcium gluconate result in complex metabolic effects, which may be classified as follows:

(*a*) An increase of plasma and urinary calcium (Nordin and Fraser, 1956), which effect is reduced in osteomalacia where calcium is retained by combination with osteoid matrix; (*b*) a decrease of urinary phosphate and diminished phosphate clearance (Nordin and Fraser, 1954); (*c*) osmotic diuresis with increased excretion of sodium, chloride, and potassium (Freedman *et al.*, 1958; Levitt *et al.*, 1958); (*d*) increased organic acid excretion. Excretion of gluconic acid is obviously directly related to the gluconate infusion. This excess urinary anion is excreted as sodium and potassium salts, and partially accounts for the increased loss of these cations after the infusion. The present observations suggest that any increase

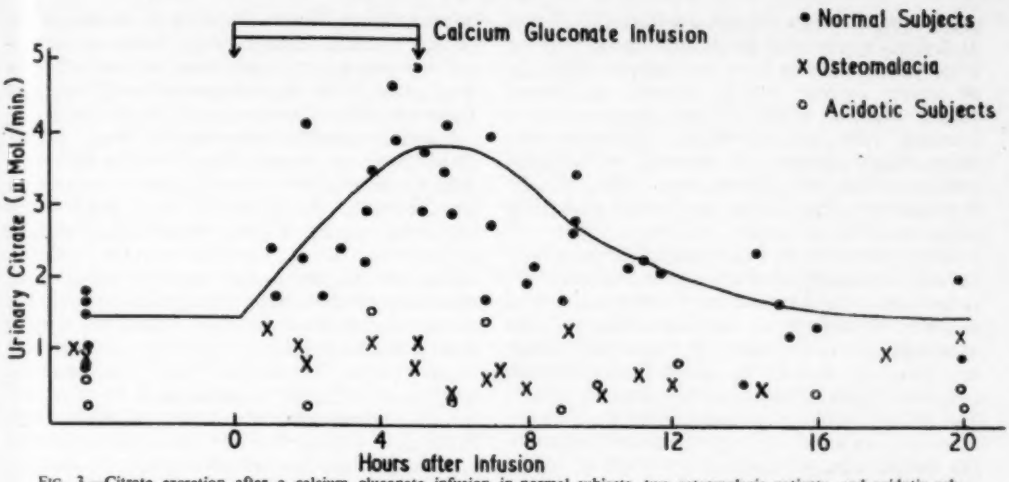


FIG. 3.—Citrate excretion after a calcium gluconate infusion in normal subjects, two osteomalacic patients, and acidotic subjects. The continuous line gives the mean excretion in the normal subjects. Citrate output is abnormally low both in acidosis and in the cases of osteomalacia.

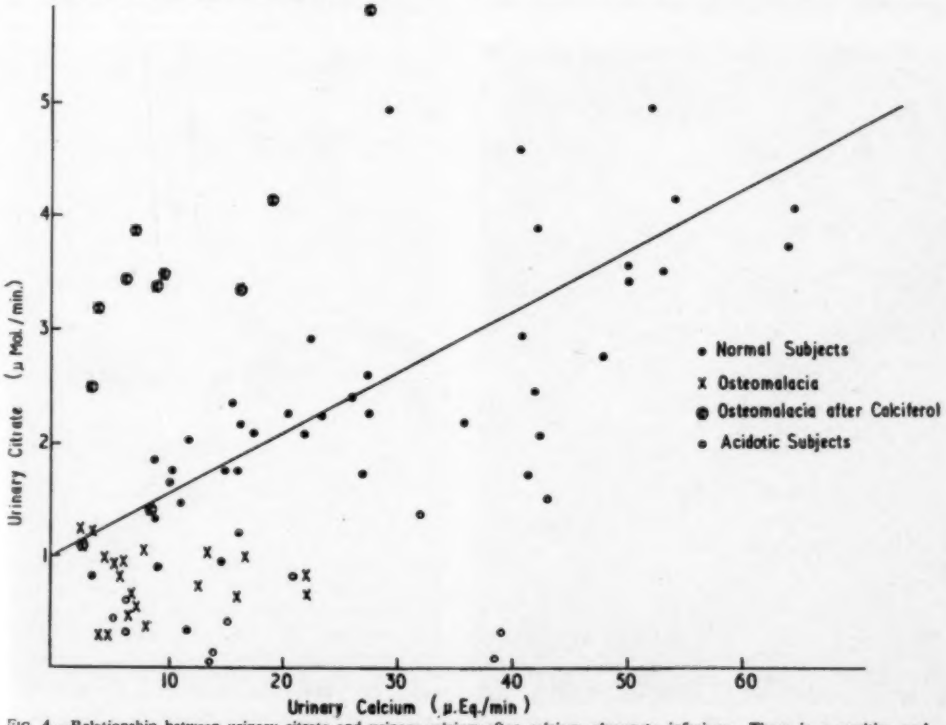
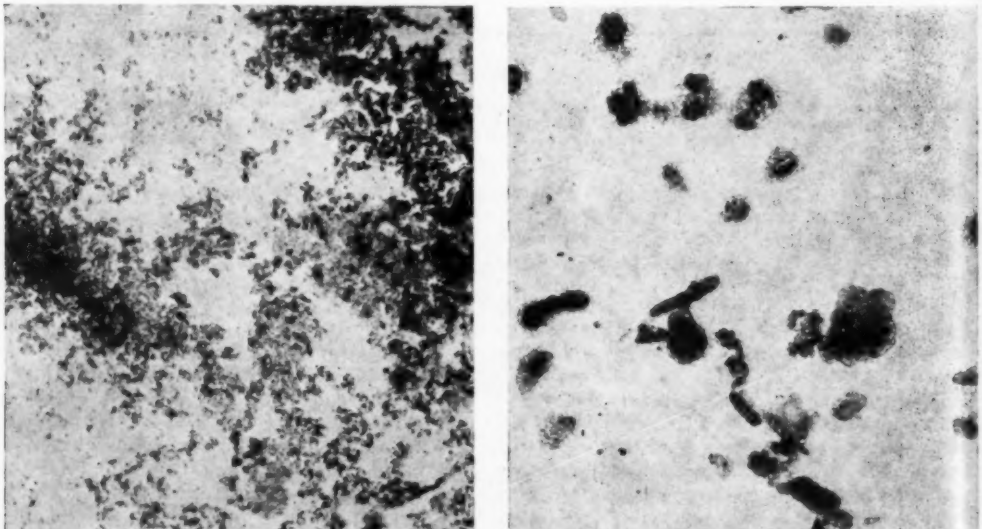


FIG. 4.—Relationship between urinary citrate and urinary calcium after calcium gluconate infusions. There is a positive and significant correlation between urinary calcium and citrate in both normal subjects and in a case of osteomalacia treated with calciferol. In acidotic subjects and in cases of untreated osteomalacia, urinary citrate is abnormally low. The straight line gives the calculated regression line in normal subjects.

of urinary citrate in normal subjects (Figs. 3 and 4) is directly related to the hypercalciuria. In the osteomalacic patients there was only a slight rise of urinary calcium and no increase in urinary citrate. Acidosis always reduces citrate excretion (Östberg, 1931 ; Clarke, Evans, MacIntyre, and Milne, 1955) and here the influence of abnormal acid-base balance overwhelmed that of the hypercalciuria, there being no increase of urinary citrate with rise of urinary calcium.

Shorr, Almy, Sloan, Taussky, and Toscani (1942) have previously reported a positive correlation between urinary calcium and citrate when excretion is modified by changes in calcium intake. Their data from a case of primary hyperparathyroidism are given in detail, the correlation coefficient between urinary calcium and citrate being +0.79. The quoted values of urinary citrate were higher than in our normal subjects, but comparable with the treated case of osteomalacia (Fig. 4). The most probable explanation of this correlation is that the calcium citrate chelation complex is less completely reabsorbed by the renal tubules than either calcium ions or free citrate. A rise of the urinary calcium level therefore tends to increase urinary citrate, and, vice versa, citrate infusions may cause a rise of urinary calcium (Chang and Freeman, 1950 ; Freeman and Chang, 1950).

Systemic acidosis enhances the renal tubular reabsorption of citrate filtered at the glomeruli, with a consequent reduction in the urinary citrate loss (Clarke *et al.*, 1955). The free citrate within the fluid in the tubular lumen falls, causing dissociation of the calcium citrate chelation complex. In this case urinary citrate may therefore be low despite hypercalciuria. The administration of physiological doses of vitamin D to normal or rachitic rats leads to an increased urinary output of citric acid (Bellin and Steenbock, 1952), and to higher concentrations of citric acid in the blood and the bones (Carlsson and Hollunger, 1954). Harrison and Harrison (1952) showed that serum and urinary citrate levels in rachitic infants were increased by vitamin D therapy. The present results suggest that the changes in urinary citrate may precede a rise of



a *b*

FIG. 5.—Precipitates of calcium phosphate in alkaline urines.
5*a*.—After sodium bicarbonate and with increased urinary citrate. 5*b*.—After acetazolamide causing decreased urinary citrate. Passage of the large angular crystals after acetazolamide was accompanied by severe strangury. ×36 in both cases.

the urinary calcium level. De Luca, Gran, and Steenbock (1957) have explained the action of vitamin D on citrate by showing an effect at the enzymatic level of metabolism. Homogenates of rat kidneys from animals receiving excess calciferol showed a 24% decrease in the rate of citrate oxidation by partial inhibition of the activity of iso-citrate dehydrogenase. Changes in urinary citrate due to calciferol may therefore be secondary to alteration of levels of renal tissue citrate.

The interrelations of urinary citrate and calcium may be of direct physiological importance in the prevention of nephrocalcinosis and the formation of renal calculi. Citrate is the most efficient natural calcium chelating agent in urine. Tricarboxylic acids chelate calcium more strongly than dicarboxylic or monocarboxylic acids (Joseph, 1946), and citric acid itself is more effective than the closely related tricarballylic acid (Schubert and Lindenbaum, 1950). Urinary citrate has been reported to be low in cases of renal calculi (Kissin and Locks, 1941; Scott, Huggins, and Selman, 1943), but this may partly be due to bacterial destruction of urinary citrate from associated urinary infection (Conway, Maitland, and Rennie, 1949). Citrate excretion is high in alkalosis (Evans *et al.*, 1957), and therefore the tendency to precipitation of calcium phosphate in akaline urine is reduced. Liability to nephrocalcinosis or stone formation would be expected in states of systemic acidosis with reduced urinary citrate accompanied by an abnormally alkaline urine. This combination occurs in renal tubular acidosis and after inhibition of renal carbonic anhydrase. Renal tubular acidosis is commonly associated with nephrocalcinosis. Efficient therapy by administration of alkali causes a rise of the previously low urinary citrate to normal levels (Bauld, MacDonald, and Hill, 1958). Prolonged treatment with acetazolamide greatly reduces urinary citrate (Clarke *et al.*, 1955), and may be complicated by renal stone formation (Gordon and Sheps, 1957), or ureteric obstruction from crystalluria (Yates-Bell, 1958). A more common and less serious toxic symptom in male patients is strangury from the presence of large crystals of calcium phosphate (Fig. 5). Urinary citrate both increases the solubility of calcium phosphate by chelation and prevents the formation of large angular crystals. Calcium phosphate is therefore usually precipitated as a fine amorphous debris which causes no urethral irritation.

Summary

Infusions of calcium gluconate cause a considerable increase of urinary organic acid. This is chiefly due to excretion of the infused gluconate, an average of 72% being excreted in the urine.

In normal subjects there is also a smaller rise of urinary citrate which parallels the rise of urinary calcium. In acidotic subjects and in cases of untreated osteomalacia, basal excretion of citrate is low and there is little or no increase of urinary citrate after the infusion. In one case of osteomalacia, calciferol treatment caused a rise of urinary citrate before the urinary calcium level was significantly increased.

The implications of the urinary calcium and citrate interrelationship in the precipitation of calcium phosphate and development of nephrocalcinosis and renal calculus are discussed.

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DIABETES INSIPIDUS COMPLICATING TOTAL ADRENALECTOMY

BY

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(RECEIVED FOR PUBLICATION MAY 10, 1959)

Diabetes insipidus is seldom complicated by other hormonal defects. As far as we know only one patient with diabetes insipidus and adrenal insufficiency has previously been described (Skillern, Corcoran, and Scherbel, 1956), although a number of patients with loss of both anterior and posterior pituitary function have been reported (Leaf, Mamby, Rasmussen, and Marasco, 1952; Engstrom and Liebman 1953; Lipsett and Pearson, 1957). Because of the difficulties encountered in the management of combined diabetes insipidus and adrenal insufficiency, the following case is recorded.

Case Report

In 1947 a married woman, aged 54, developed carcinoma of the left breast, which was treated by radical mastectomy and local irradiation. She remained well until March, 1957, when malaise, loss of weight, and widespread bone pain developed. There was no local recurrence, but radiographs revealed extensive pulmonary and osseous metastases, particularly involving the femora, pelvis, and skull with erosion of the sella turcica. After oophorectomy and total adrenalectomy, performed by Sir Stanford Cade, the patient's post-operative course was uneventful and she was discharged in May, 1957, taking 50 mg. cortisone daily by mouth. She felt well enough to convalesce abroad, but a month later noticed a recurrence of malaise with increasing thirst and polyuria. Despite increasing the dose of cortisone to 125 mg. daily and taking extra salt, she deteriorated rapidly, became comatose and was admitted to hospital again on June 23, 1957 (Day 1, Fig. 1).

On entry she was mentally confused and extremely dehydrated. The pulse was 120/mm. and of small volume with a blood pressure of 100/70 mm. Hg compared with 135/80 mm. Hg the previous month. The haemoglobin level was 19.2 g.% in contrast to a previous value of 10.8 g.%, and the serum electrolytes were as shown in the following table.

	On Entry in June, 1957	May, 1957
Sodium ..	177 mEq/l.	146 mEq/l.
Potassium ..	3·3 ,,	4·9 ,,
Chloride ..	140 ,,	108 ,,
Total protein ..	7·9 g.%	7·0 g.%

The history of thirst and polyuria together with the dehydration and haemoconcentration indicated diabetes insipidus, and treatment with vasopressin ("pitressin") was started. The serum potassium concentration was low in contrast with the high serum sodium and chloride levels, and an electrocardiogram showed changes of moderate hypokalaemia. This hypokalaemia was attributed to the large doses of cortisone which the patient had been taking. Over the next 12 hours 3.5 l. of fluid and 90 mEq of potassium were given orally with an additional 125 mg. cortisone, making a total of 175 mg. on this day. The patient improved rapidly and became mentally alert. The dehydration disappeared and the blood pressure became normal with weight gain, a positive fluid balance, and a fall in plasma protein and serum sodium levels. By the third day her clinical state was satisfactory and the extracellular fluid volume, as measured by the bromide space (Nicholson and Zilva, 1957), had expanded by nearly 4 litres to 16.5 litres, correlating closely with the cumulative fluid gain (Fig. 1). At this stage the bromide space was probably a reliable measure of the extracellular fluid volume and was within the normal range for the patient's height and weight.

During this time she had received 1 ml. "pitressin" tannate in oil intramuscularly each day (5 pressor units/ml.), but from the fourth day onwards she deteriorated unexpectedly so that by the eighth day the weight and bromide space had fallen to the same levels as on entry, despite increasing the "pitressin" to 1.5 ml. daily on Day 7. It was then discovered that aqueous "pitressin" (20 pressor units/ml.) had been given in error for the oily suspension of "pitressin" tannate, and, despite a sixfold increase in dosage, had produced no effective overall antidiuretic response. Fig. 1 shows that when this error was corrected the diabetes insipidus was once again controlled.

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Despite this she deteriorated, and by Day 10 was confused, muscles were twitching, and she complained of cramps. The serum sodium level had fallen to 127 mEq/l. and the urinary loss of sodium on this day was 336 mEq. Since admission the dose of cortisone had been reduced stepwise and maintained at 75 mg. daily from the fourth day onwards. The progressive sodium loss despite hyponatraemia indicated that the sodium-retaining effect of this dose of cortisone was inadequate to maintain a normal sodium balance, and the clinical picture resembled that of acute adrenal insufficiency. During this period (Days 9–15) the bromide space expanded rapidly to 33 l., a value much exceeding the extracellular fluid volume and approximating to the probable total body water. This suggested that bromide had entered the cells, a phenomenon which has been observed in other patients with severe adrenocortical insufficiency or other conditions likely to affect the permeability of cell membranes. At this time the serum bicarbonate, calcium, and urea levels were all normal.

Despite increasing the daily dose of cortisone to 100 mg. on Days 10 and 11, the serum sodium level continued to fall to 113 mEq/litre on Day 13, when 0.5 mg. fluorohydrocortisone daily was started. This potent sodium-retaining hormone caused a striking improvement, and by Day 16 the patient was well again. A further dose of radiobromide was given, and the normal figures for the bromide space on Days 17–19 suggested that adrenocortical substitution therapy was now adequate.

Thereafter she remained well on 75 mg. cortisone and 0.25 mg. 9α - fluorohydrocortisone daily by mouth, with 2–4 units of "pitressin" tannate in oil every other day. She was discharged on Day 20 and returned home, where she led an active life doing her own housework

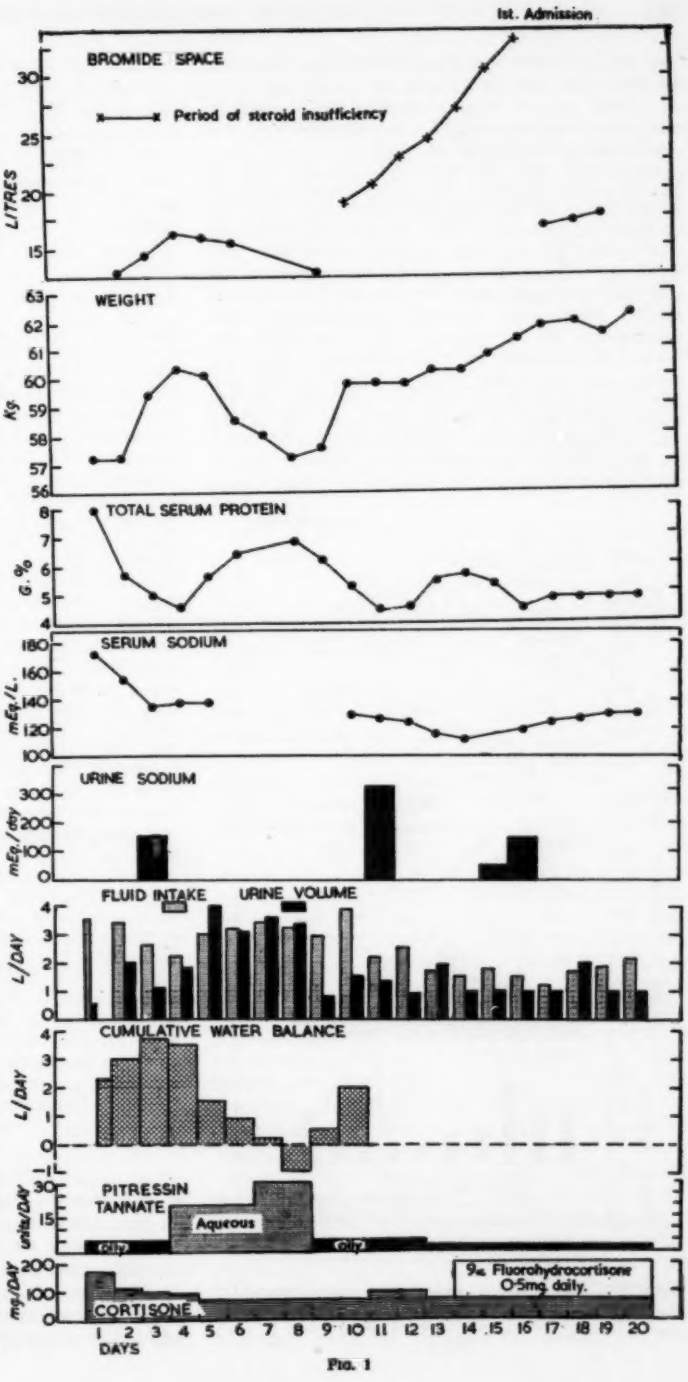

Fig. 1

and driving a car. The serum sodium level fluctuated between 130 and 156 mEq/l., and a 24-hour assay of urinary steroid excretion showed that the supplements were well absorbed (17-ketosteroids 4.9 mg. and 17-hydroxycorticosteroids 25.3 mg.).

In October, 1957, attacks of headache and vomiting began, and in February, 1958, an enlarging metastasis in the left femur caused severe pain which led to the patient's readmission. The diabetes insipidus was well controlled and the cerebral symptoms were attributed to metastases. During this admission the effect of varying the dose of "pitressin" tannate was studied while she received a constant steroid supplement of 75 mg. cortisone and 0.25 mg. fluorohydrocortisone, and a relatively constant sodium intake of 200 mEq daily. Fluid intake was unrestricted and there was no vomiting. At the beginning of this period of observation the patient was not dehydrated and the bromide space of 14.5 l. on Day 2 (Fig. 2) was within normal limits. Initially the dose of "pitressin" tannate in oil was 0.5 units

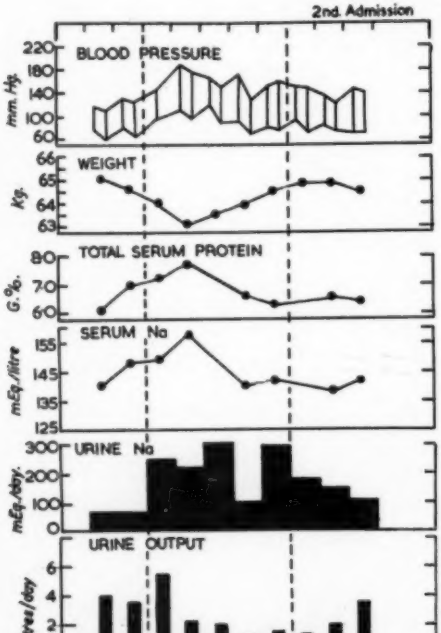

FIG. 2

TABLE I

URINARY EXCRETION OF WATER AND ELECTROLYTES IN RELATION TO "PITRESSIN" DOSAGE

	"Pitressin" Tannate in Oil (Units/day)	Mean Daily Excretion			
		Sodium (mEq/day)	Potassium (mEq/day)	Chloride (mEq/day)	Urine (ml./day)
Period 1 (2 days)	½	74	93	162	3,730
Period 2 (5 days)	2	234	100	236	2,375
Period 3 (3 days)	1	151	100	188	2,340

daily, and the average urine volume on Days 1 and 2 was 3,730 ml. (Table I), indicating that the diabetes was not completely controlled. On Day 3 the dose of "pitressin" tannate was increased to 2 units daily. The effect of this was delayed but was followed by an increase in blood pressure and in urinary sodium excretion. On the first day of this dosage the urine volume increased to 5 l. and was associated with haemoconcentration, as shown by the rise in serum sodium and plasma protein concentrations on Days 3 and 4. On the fourth day the urine volume fell and thereafter better control of the diabetes insipidus was obtained, judging from the urine volume, gain in weight, and return of the serum sodium and plasma protein levels to normal. Reducing the dose of "pitressin" tannate to 1 unit daily on Day 8 was accompanied by a gradual fall in sodium excretion and a gradual increase in urinary volume, suggesting that this dose did not quite control the diabetes insipidus. The pattern of these responses showed that there was some delay in the absorption of "pitressin" tannate in oil from the site of injection. Thus the maximum effect of 2 units daily was not evident for 24 to 48 hours and the increased diuresis caused by halving the dose of "pitressin" on Day 8 was delayed for a similar period.

On Day 2 the total exchangeable sodium, measured with radiosodium, was 3,450 mEq (54 mEq/kg. body weight), an abnormally high figure. The total exchangeable potassium at the end of the study was abnormally low at 1,280 mEq (21 mEq/kg. body weight). These findings suggested that the amount of adrenocortical substitution therapy was excessive, and the dose of fluorohydrocortisone was reduced after Day 10 to 0.1 mg. daily. Later the dose of cortisone was increased to 150 mg. daily, but this did not influence the response to a constant amount of "pitressin" tannate.

Discussion

The modern treatment of advanced carcinoma of the breast may so prolong life that the rarer complications of metastases, such as diabetes insipidus, may occur more frequently. Retrospective study of this patient's fluid chart after adrenalectomy shows urine volumes of 3 to 6 litres daily and a negative fluid balance, indicating the

onset of diabetes insipidus although she did not then complain of thirst.

As a result of adrenalectomy and destruction of the neurohypophysis, the hormonal control of both salt and water balance was lost. Thus initially, whilst receiving large doses of cortisone but deficient in antidiuretic hormone, sodium was retained despite the polyuria, and the clinical picture was that of water depletion. At this stage the patient resembled the case described by Engstrom and Liebman (1953) in whom diabetes insipidus complicated anterior pituitary failure. However, by Day 3 (Fig. 1) the fluid deficit had been corrected with " pitressin " tannate in oil, but thereafter (Days 4–7) progressive sodium and water depletion occurred as a result of the reduced dose of cortisone and the unsuitable preparation of vasopressin. The loss of water was shown by the fall in weight, the low bromide space, and the haemoconcentration on Day 9. When " pitressin " tannate was re-started water balance was restored, but by Day 10 severe salt depletion was revealed with a serum sodium of 127 mEq/litre and a urinary sodium loss of 336 mEq on this day, indicating insufficient sodium-retaining hormone. Only when sodium retention was increased with fluorohydrocortisone was correct salt and water balance achieved. The wide fluctuation in serum sodium levels when the patient was an out-patient shows that this balance was precarious, but her satisfactory clinical state suggests that a reasonable compromise was obtained.

Influence of Cortisone on Polyuria in Diabetes Insipidus

Several reports have shown that the thirst and polyuria of experimental diabetes insipidus are reduced if the anterior pituitary is destroyed or the adrenal glands removed (Biggart and Alexander, 1939; Ingram and Winter, 1938; Gaunt, Birnie, and Eversole, 1949). This fall in urine volume, despite continuing lack of antidiuretic hormone (ADH), is probably due to several factors, particularly a decrease in the solute-load requiring excretion and the reduction in glomerular filtration rate. Leaf and his associates (1952) showed in a patient with loss of both anterior and posterior pituitary function that lack of ADH, as shown by an inability to elaborate a concentrated urine, persisted, and the absence of polyuria depended upon the reduction in solute load. When this was increased the polyuria recurred, and when the solute load was kept constant cortisone had no effect on water excretion. These findings suggest that the influence of cortisone on the polyuria was due to its general metabolic effect which led to an increase in solute excretion.

Cortisone also increases urine flow in patients with adrenocortical insufficiency by increasing the glomerular filtration rate. Burston and Garrod (1952) found in Addisonian patients that the rate of urine flow up to 5 ml./min. depended upon the glomerular filtration rate, and Skillern *et al.* (1956) also concluded in their patient with Addison's disease and diabetes insipidus that the diuresis induced by hydrocortisone was attributable to an increase in the glomerular filtration rate.

It has also been suggested that cortisone may antagonize the action of antidiuretic hormone on the renal tubules (Gaunt *et al.*, 1949), but in our patient doubling the dose of cortisone from 75 to 150 mg. daily did not influence the requirement of " pitressin " tannate.

Influence of Antidiuretic Hormone on Electrolyte Excretion

There are many conflicting reports on the effect of " pitressin " on sodium and chloride excretion depending partly on species variation and partly on the degree of preliminary hydration. In normal subjects " pitressin " does not affect sodium output (Black and Thomson, 1951) though at low rates of urine flow a slight fall in sodium excretion may occur (Murphy and Stead, 1951). Under artificial conditions, when the extracellular fluid volume is expanded by pitressin a delayed sodium diuresis results (Leaf, Bartter, Santos, and Wrong, 1953). This is accompanied by potassium retention and is attributed to secondary inhibition of aldosterone production (Liddle, Bartter, Duncan, Barber, and Delea, 1955; Wrong, 1956).

In the case of anterior and posterior pituitary failure reported by Engstrom and Liebman (1953) " pitressin " had no effect on sodium excretion, and, though Leaf *et al.* (1952) comment on the natriuresis produced by " pitressin " in their patient, this effect took three days to become fully apparent. It was probably not a direct effect of " pitressin " but due to secondary inhibition of sodium-retaining mechanisms.

The variation in urinary sodium and chloride excretion with " pitressin " dosage in the patient being discussed, on fixed doses of adrenocortical steroid, is therefore surprising. There is some day-to-day variation within the periods and the findings must be viewed with some suspicion. The diet, however, was constant and potassium excretion was fairly uniform throughout. She was

adequately hydrated at the beginning of the investigation and the dose of cortisone and 9α-fluorohydrocortisone seemed adequate. Some support to the findings is given by the fact that after adrenalectomy she was adequately maintained on 50 mg. cortisone orally, whereas, when receiving "pitressin," double this dose did not prevent sodium depletion (Days 10–11, see Fig. 1).

The increase in sodium excretion was not delayed, was not accompanied by potassium retention, and therefore cannot be explained by changes in sodium-retaining hormones. In the presence of severe renal disease sodium excretion may vary with the blood pressure as a result of changes in renal plasma flow. In this patient renal function was normal (blood urea 27 mg.% on Day 2), and this mechanism seems unlikely. Furthermore, Murphy and Stead (1951) have shown that "pitressin" in physiological doses does not affect renal plasma flow or glomerular filtration. It may be that the increased sodium excretion is a direct effect of "pitressin," normally masked by simultaneous changes in sodium-retaining mechanisms, an explanation which is physiologically acceptable but without supporting experimental evidence.

This combination of endocrine defects proved difficult to treat, especially as sodium depletion may be masked by the diabetes insipidus. If a satisfactory preparation of "pitressin" is used, water balance is readily restored and steroid dosage must then be adjusted according to the serum electrolytes and urinary sodium excretion.

Summary

A patient* is reported in whom diabetes insipidus developed following total adrenalectomy for carcinomatosis.

During treatment syndromes of sodium and water depletion were seen which varied independently.

Aqueous "pitressin" was of no value in treatment even in high dosage, whereas oily "pitressin" tannate was very satisfactory.

An unusually high dosage of steroid was required for maintenance.

Sodium excretion appeared to vary with "pitressin" dosage.

We wish to thank Sir Stanford Cade for permission to publish this case. We are indebted to Dr. R. I. S. Bayliss and Professor N. F. Maclagan for their advice in the preparation of this paper. The illustrations were prepared by Miss Jeanette Pirkis.

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* Since this paper was written, the patient has died. Unfortunately no necropsy could be performed.

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RADIOIODINATED TRIOLEIN IN MALABSORPTION STATES

BY

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In the light of the difficulties and inadequacies of established methods in the investigation of intestinal malabsorption the report by Stanley and Thannhauser (1949) that radioiodinated olive oil might provide an index of fat absorption appeared to be of considerable interest. Subsequent investigators in America, using a pure neutral fat, triolein, similarly iodinated, have reported on the studies of levels of stool, urine, and blood radioactivity in various diseased states. Variations in technique and in the criteria for interpretation of results have resulted in conflicting claims regarding the relative value of blood and stool analysis after an oral dose of radioiodinated triolein.

The results therefore are reported here of an investigation undertaken to test the hypothesis put forward by the group of workers from the Duke University School of Medicine, North Carolina (Ruffin, Shingleton, Baylin, Hymans, Isley, Sanders, and Sohmer, 1956; Ruffin, Keever, and Chears, 1957; Shingleton, Baylin, Isley, Sanders, and Ruffin, 1957a; Shingleton, Isley, Floyd, Sanders, Baylin, Postlethwait, and Ruffin, 1957b; Isley, Sanders, Baylin, Sharpe, Hymans, Ruffin, Shingleton, and Wilson, 1957; Ruffin, Keever, Chears, Shingleton, Baylin, Isley, and Sanders, 1958) that an "invariable inverse relationship exists between the radioactive material in blood and stool" after an oral dose of radioiodinated triolein. The opportunity was also taken to study simultaneously the urinary radioactivity.

Materials and Methods

Patients.—Patients in the present study consisted of two broad groups:

Control Series.—This consisted of 32 "normal" ward patients with no clinical evidence of gastrointestinal or renal disease and six known cases of idiopathic steatorrhoea. The diagnosis of steatorrhoea had been established on clinical and biochemical evidence, including a conventional fat-balance study. All six patients had been on treatment with a gluten-free diet, with or without folic acid, for periods exceeding one year. Four were in complete clinical and haematological remission as evinced by their bowel rhythm, stool consistency, nutritional status, and blood levels. The remaining two patients had persistent but variable morning diarrhoea with some bowel urgency.

"Blind" Series.—Fourteen patients at another hospital formed a "blind" series. Their diagnosis was known to only one of us (R. H.), who carried out the tests in these cases and submitted the specimens to the other (S. D. M.) for assay of radioactivity and an opinion. Ten of the patients in this series were "normal" and the remaining four were cases of idiopathic steatorrhoea diagnosed according to criteria previously mentioned. All but one had been on treatment with a gluten-free diet, with or without folic acid, for periods exceeding four weeks. Two of these patients, the longest on therapy, namely six and eight months, were in complete clinical and haematological remission.

Test Meal.—The test meal was prepared with radioiodinated triolein† after the procedure outlined by Ruffin *et al.* (1956) with minor modifications (Fig. 1). Two emulsions of arachis oil with equal volumes of water were prepared using commercial Tween 80 as an emulgent. One of these, emulsion "A," contained the radioiodinated triolein.

TEST MEAL OF ^{131}I TRIOLEIN

Hot Emulsion (A)	*Cold Emulsion (B)*
1 ml. ^{131}I triolein	200 ml. arachis oil
49 ,, arachis oil	200 ,, water
50 ,, water	15 ,, Tween 80
4 ,, Tween 80	

Test meal = Vol. of A with 20–25 μc. ^{131}I
+ Vol. of B ——→
Final vol. of 1 ml./kg.

FIG. 1

The test meal consisted of a volume of emulsion "A," containing 20–25 μc. of ^{131}I with sufficient volume of emulsion "B" to give a final volume of 1 ml./kg. body weight, i.e., approximately 0.5 ml. arachis oil/body weight.

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†Supplied by Radiochemical Centre, Amersham.

Procedure.—Lugol's iodine, 10 minims t.i.d., was given by mouth for two days before the test to minimize thyroidal uptake of ^{131}I which becomes split off *in vivo* after absorption of radioiodinated triolein.

After an overnight fast the patient ingested the test meal and the washings from two rinses of the container with tepid water. Residual activity in the container was extracted with petroleum ether and this was taken into consideration in the final assessment of total activity actually administered.

Venous blood samples were then withdrawn into oxalated containers at hourly intervals for six hours. Urine was collected in two 24-hour periods following the test dose. Stools were collected for 72 hours in 500 ml. waxed cardboard cartons.

Radioactivity was measured on 2 ml. and 5 ml. aliquots of whole blood and urine, respectively, in a well-type scintillation counter. Suitable dilutions of stock standard solutions, prepared simultaneously with the test meals, were also assayed. Results were expressed as percentage of dose administered per litre of blood and percentage of dose excreted in each 24-hour and cumulative 48-hour period from the test meal.

Stool collections were assayed for radioactivity by placing the cartons in a large well cut out in a lead shield placed over a thallium activated sodium iodide scintillation crystal. Suitable aliquots of the stock standard solutions were assayed by suspension in a 10% gelatin solution in similar cartons. Activity in stools was expressed as percentage of dose administered.

Results

Blood Tests.—The observed levels of radioactivity in the 32 "normal" patients with the relevant statistical ranges are shown in Table I and Fig. 2. Results of duplicate blood tests on two patients, shown in Fig. 3, attest to the reproducibility of the characteristic pattern of blood radioactivity in the individual cases.

TABLE I

BLOOD RADIOACTIVITY AS PERCENTAGE OF DOSE PER LITRE OF RADIOIODINATED TRIOLEIN IN 32 NORMAL CONTROLS

	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	6 Hours
$\bar{x}$	0·74	1·60	2·46	2·84	2·77	2·71
σ	±0·4	±0·54	±0·65	±0·70	±0·75	±0·72
$\bar{x}\pm\sigma$	0·34–1·14	1·06–2·14	1·81–3·11	2·14–3·54	2·02–3·52	1·99–3·43
$\bar{x}\pm2\sigma$	0–1·54	0·52–2·68	1·16–3·76	1·44–4·24	1·27–4·37	1·27–4·15

$\bar{x}$ = Mean. σ = Standard deviation.

In Fig. 4 the levels of radioactivity in the six control cases of steatorrhoea are superimposed on the graph of mean levels and one standard deviation range observed in the "normal" control patients. It is apparent that two curves in this group are indistinguishable from those of the "normal" controls.

Fig. 5 shows the blood radioactivity in four cases of steatorrhoea in the "blind" series. The remaining 10 "normal" patients in this series showed curves indistinguishable from those of the normal control subjects and are therefore not shown in the figure.

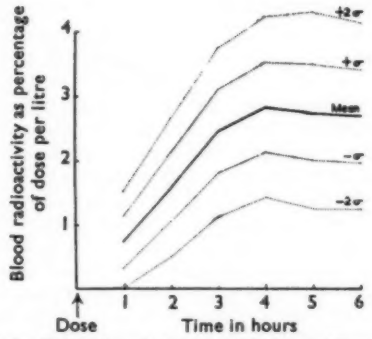

FIG. 2.—Radioiodinated triolein test in 32 "normal" controls.

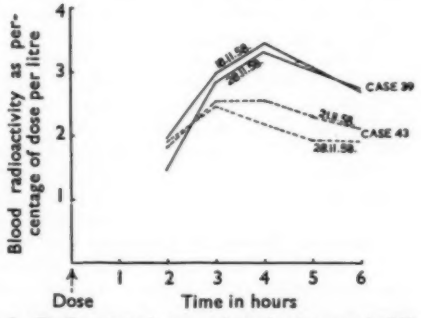

FIG. 3.—Duplicate tests in two patients showing reproducibility of blood curves.

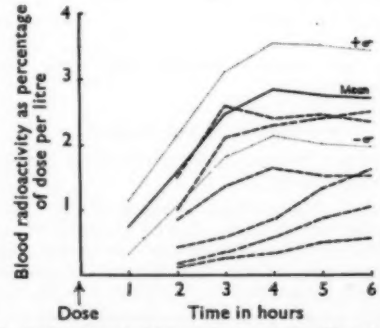

FIG. 4.—Radioiodinated triolein test in six controls with steatorrhoea.

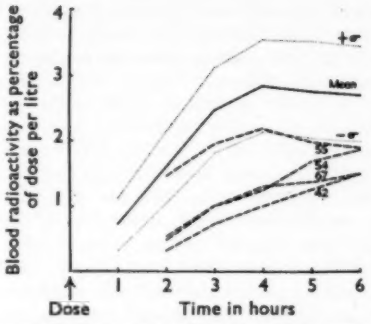

FIG. 5.—Radioiodinated triolein test in the "blind" series of four cases of steatorrhoea.

Three of the patients with steatorrhoea can be readily identified, but the fourth case with levels of radioactivity similar to those of the "normal" control patients was missed on this analysis.

Urine Tests.—Table II shows the levels of radioactivity in the individual 24- and cumulative 48-hour urine collections with the relevant statistical ranges as observed in 24 of the "normal" control patients in whom the urinary radioactivity was studied.

TABLE II

URINARY EXCRETION OF RADIOACTIVITY AS PERCENTAGE OF DOSE OF RADIOIODINATED TRIOLEIN IN 24 NORMAL CONTROLS

Time (hr.)	Mean	Standard Deviation (σ)	Mean $\pm\sigma$	Mean $\pm 2\sigma$
0–24 (16 cases)	44·4	±12·37	32·03–56·77	19·66–69·14
24–48 (16 cases)	16·5	± 6·3	10·2–22·8	3·9–29·1
0–48 (24 cases)	55·3	±12·9	42·4–68·2	29·5–81·1

Tables III and IV show the excretory pattern in the six control patients with steatorrhoea and all the 14 patients in the "blind" series, respectively.

TABLE III

URINARY EXCRETION OF RADIOACTIVITY AS PERCENTAGE OF DOSE OF RADIOIODINATED TRIOLEIN IN CONTROLS WITH STEATORRHOEA

Patient	0–24 hr.	24–48 hr.	0–48 hr.
32	47·5	16·9	64·4
34	o^+ 14·2 o^{++}	15·9	o^+ 30·1
36	—	—	—
52	—	—	o^+ 26·0 o^{++}
37	34·8	o^+ 8·0	42·8
50	o^+ 9·69 o^{++}	o^+ 0·55 o^{++}	o^+ 10·24 o^{++}

o^+ Abnormal at 1 S.D. Range of normal excretion.
o^{++} Abnormal at 2 S.D. Range of normal excretion.

TABLE IV

URINARY EXCRETION OF RADIOACTIVITY AS PERCENTAGE OF DOSE OF RADIOIODINATED TRIOLEIN IN "BLIND" SERIES

Patient	0–24 hr.	24–48 hr.	0–48 hr.
42	o^+ 22·4	10·4	o^+ 32·8
43	40·2	11·25	51·45
44	o^+ 29·4	17·8	47·2
45	36·2	17·9	54·1
46	58·2	19·05	77·25
53	47·4	22·9	70·3
54	47·2	16·3	63·5
55	66·0	18·8	84·8
57	—	—	—
61	—	—	—
63	—	—	—
64	65·2	20·9	86·1
65	68·4	o^+ 4·14	72·54
67	45·5	12·2	57·7

o^+ = Abnormal at 1 S.D. range of normal excretion.
o^{++} = Abnormal at 2 S.D. range of normal excretion (none).
Numbers set in bold type show patients with steatorrhoea.

Those considered abnormal at the various statistical levels of accuracy are delineated in the tables and it is apparent that the urine tests form an even less satisfactory index than the blood tests for distinguishing the abnormal patients.

Stool Tests.—Table V shows the results of stool radioactivity analysis in the entire series of cases. Twenty of the 32 "normal" patients on whom stool tests were successfully completed show a statistical range of excretion of 0 to 2.8%.

TABLE V

RADIOACTIVITY AS PERCENTAGE OF DOSE IN 72 HOURS. STOOL COLLECTIONS AFTER RADIOIODINATED TRIOLEIN

Subjects	No.	Observed Values	Mean $\pm 3\sigma$
Control cases:			$\frac{(0{\cdot}88 \pm 3 \times 0{\cdot}64)}{0–2{\cdot}8}$
"Normal" ..	20	0·13–2·2	
Steatorrhoea ..	6	4·1–37·6	
"Blind" series:			$\frac{(0{\cdot}68 \pm 3 \times 0{\cdot}69)}{0–2{\cdot}75}$
"Normal" ..	10	0–2·35	
Steatorrhoea ..	4	44·2	
		5·8	
		0·9	
		4·3	

σ = Standard deviation.

The six control patients with steatorrhoea excreted radioactivity in amounts varying from 4.1 to 37.6% of the dose, which distinguished all six patients from the normal controls.

Radioactivity in the stools of the 10 "normal" patients in the "blind" series, namely 0 to 2.75%, was comparable to that of the "normal" patients in the control series.

The excretion of radioactivity in the stools of four patients with steatorrhoea in the "blind" series is shown individually and of these three

can be separated from the "normal" controls. The fourth patient excreted radioactivity in amounts consistent with a satisfactory fat absorption. This was the same patient who was missed in the blood tests.

Discussion

The use of ^{131}I as a tag for triolein depends on the assumption that the chemical bond between the two substances is stable *in vitro* during the process of digestion and absorption. That this assumption is probably correct is suggested by the findings of several workers who have studied the metabolism of radioiodinated triolein in man and in laboratory animals (Hoffman, 1953; Shingleton, Wells, Baylin, Ruffin, and Sanders, 1955; McCandless and Zilversmit, 1955; Ruffin *et al.*, 1956; Turner, 1956; Malm, Reemtsma, and Barker, 1956; Beres, Wenger, and Kirsner, 1957; Reemtsma, Malm, and Barker, 1957; Van Handel and Zilversmit, 1957; and Duffy and Turner, 1958). We have confirmed that the major part of ^{131}I is in the lipid fraction of blood in the early stages of absorption and cannot be dialysed but can be precipitated with trichloracetic acid. Radioactivity in the precipitate is not water soluble but can be extracted with fat solvents. We have also been able to confirm that the stool radioactivity is not dialysable but can be extracted with ether. Radioiodinated triolein is stable after six hours' incubation with gastric juice, 25% HCl, and trichloracetic acid. The emulsion used for the test meals was found to be stable when examined over a period of four weeks (unpublished data).

Variations in the amount and nature of vehicles used for the administration of radioiodinated triolein, insistence on a fasting state of the stomach before the test meal or otherwise, and the diverse criteria used in the evaluation of results manifest in the various reported investigations on the use of radioiodinated triolein, make it difficult to compare the results of any two series with confidence. The method suggested by Ruffin *et al.* (1956) for the test procedure seems to be least subject to criticism. It ensures the use of a known amount of nutrient of constant composition, namely 0.5 ml. of arachis oil per kilogram body weight, and ensures a fasting state of the stomach before and during the initial six hours of the test, i.e., the period of the blood test. The need for prior preparation of the patient with Lugol's iodine has recently been disputed by Grossman and Jordan (1958). They found that the omission of this precaution "does not introduce any significant difference in the blood or faecal levels of radioactivity." Using such prior preparation with iodine, however, we have found no significant difference in the levels of radioactivity over the neck and thigh in three of the normal control patients examined during the six-hour period of the blood test. Malm *et al.* (1956) report no appreciable uptake by the thyroid gland in patients so prepared. The use of preliminary iodine medication seems desirable, at least to protect the thyroid from any unnecessary irradiation, however small.

The pattern of blood radioactivity observed in the "normal" control patients suggests that the blood curve is characteristic and reproducible in the same patient (Fig. 3). This is in agreement with the findings of Baylin, Sanders, Isley, Shingleton, Hymans, Johnston, and Ruffin (1955) and Beres, Wenger, and Kirsner (1957). McKenna, Bourne, and Matzko (1957) have, however, concluded otherwise, but it is suggested that their experience may have been due to the patient having breakfast before the test meal.

Of the six known cases of idiopathic steatorrhoea in the control series, only four showed results below the normal limits of minimal "absorption." The remaining two cases, even on the statistically unsatisfactory criteria of one standard deviation range of the normal levels, show curves indistinguishable from the "normal" control patients. Both these patients were in full clinical and haematological remission as, indeed, were two of the four patients with abnormal results. These results are not surprising when it is considered that the instantaneous blood radioactivity is a function of many diverse and, as yet, ill-understood factors. Thus the rate and amount of absorption, utilization, storage, and extravascular sequestration of fat, the completeness of the thyroid block, the renal clearance of free ^{131}I split off *in vivo*, and the rate of gastric emptying would all affect the observed blood levels of radioactivity. It is of interest to note that Peters and Van Slyke (1946), reviewing the data of chemical determinations of serum lipids in the post-operative state in normal subjects, observed a wide range. They suggest that this is due, in some measure, to the inclusion in most reported series of stray maximum and minimum figures obtained from ostensibly normal persons. Similar extreme readings are to be noted in almost all adequately documented series on radioiodinated triolein.

The observed levels of blood radioactivity in the control series were also assessed by alternative

criteria suggested, such as the four-hour radioactivity levels (based on the fact that most of the normal patients show peak levels of blood radioactivity at this hour), and the average of four-, five-, and six-hour levels of radioactivity (Baylin *et al.*, 1955; Ruffin *et al.*, 1956; Shingleton *et al.*, 1957a and b; Berkowitz and Sklaroff, 1957; and Kaplan, Edidin, Fruin, and Baker, 1958). In addition, as seven of our normal control subjects had peak levels of radioactivity at three hours, five at five hours, and three as late as six hours, assessment was also made in terms of peak levels of radioactivity alone. Results of these alternative assessments, however, showed no significant improvement in identification of the abnormal cases on the method described. Findings in three normal subjects of peak levels of blood radioactivity as late as the sixth hour suggested that the blood radioactivity should be studied for more prolonged periods. A small number of patients were studied for seven to eight hours, but this was not found to give any additional information of value.

Radioactivity in the urine collections was almost completely accounted for by inorganic ^{131}I, which was totally extractable by a Cl phase ion exchange resin (amberlite resin, IRA-400 (Cl), B.D.H.). The 24- and 48-hour urinary radioactivity is, therefore, even less satisfactory than the blood tests as it represents the end-result of the various processes determining the blood radioactivity as described above.

In the assessment of stool radioactivity, it was feared that errors might be introduced in the results through contamination of the stools with significant amounts of radioactivity in the urine, a contingency difficult to avoid with certainty, especially in the female patients. The more stringent levels of statistical accuracy of three standard deviation ranges were therefore adopted. With these criteria, all six patients with idiopathic steatorrhoea were readily differentiated from the "normal" control patients. It was also noted that all six patients excreted radioactivity in amounts greater than 4% of the dose. For added safety, the 4% figure was accepted as an arbitrary upper limit of normal and it was decided to regard any results between 2.8 and 4% as possibly due to urinary contamination.

The results of the control series, therefore, failed to confirm the claim put forward by the North Carolina group of workers that an "invariable inverse relationship exists between the radioactive material in blood and stool."

Conclusions were further tested in a group of 14 "blind" cases as described. In this series, however, both the blood and stool tests identified three of the four patients with idiopathic steatorrhoea. The probability of such differentiation obtaining by chance is 1 in 25, which renders the results of both tests highly significant in this group. It should be noted that the curves of normal limits of blood radioactivity are computed at one standard deviation range, which excludes as many as 16% of normal subjects at the lower end of the normal curve. Results of the blood tests are, therefore, inferior to the stool tests as concluded in the control series. The similarity in the range of faecal radioactivity in the 10 "normal" patients in the blind series and 30 similar patients in the control series would seem to suggest that the dangers of cross-contamination with urinary radioactivity can be over-emphasized when proper precautions have been taken to eliminate, or at any rate to minimize, this hazard.

The results in the fourth patient with idiopathic steatorrhoea in the "blind" series are indistinguishable from those of "normal" control subjects on either test. This patient was in complete clinical and haematological remission, as indeed were two of the remaining three who gave abnormal results. A final reassessment of fat absorption with chemical balance studies in the one patient with anomalous results was unfortunately not possible as the patient was unwilling to re-enter hospital. The exact state of fat absorption in this patient must, therefore, remain in doubt. It is of interest to note, however, that her urinary excretion of 85% of the dose over the 48-hour period was the second highest result recorded in the entire series.

Within the limits of this trial, it is concluded that the stool test with radioiodinated triolein may be of value in establishing the diagnosis of steatorrhoea. (Lubran and Pearson (1958), using a similar technique, came to the same conclusion regarding its usefulness as a screening test in this condition.) Finding low curves of blood radioactivity would suggest steatorrhoea, but normal levels do not exclude the presence of steatorrhoea. These findings are in agreement with those reported by McKenna *et al.* (1957) and Grossman and Jordan (1958). Urinary radioactivity does not provide a satisfactory diagnostic index and may in fact introduce error in the stool tests if rigid precautions to avoid cross-contamination of stools with urine are not observed.

Summary

Results of blood, urine, and stool tests with radioiodinated triolein in a control series of 32 "normal" and six known cases of idiopathic steatorrhoea under therapy are reported.

Comparable studies in a "blind" series of 10 "normal" patients and four patients with idiopathic steatorrhoea (three treated) are described.

Of the 10 cases of steatorrhoea in the series, the blood tests identified seven as abnormal (one untreated, six treated), whereas the stool tests differentiated nine cases (one untreated, eight treated). One treated case in the "blind" series, in full clinical remission, was missed on both tests, but excreted radioactivity in urine in amounts at the upper limits of the normal range. Results of urine tests were much less satisfactory.

Within the limits of this trial it is concluded that the stool tests with radioiodinated triolein may be of value in establishing the diagnosis of steatorrhoea. Finding low levels of blood radioactivity would suggest steatorrhoea (within the statistical limitations described), but normal levels do not exclude the presence of steatorrhoea. Analysis of urine for radioactivity is much less satisfactory.

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A SCREENING TEST FOR PHAEOCHROMOCYTOMA

BY

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Patients suffering from phaeochromocytomata excrete many times the normal amount of noradrenaline in the urine (Euler, 1951). Determination of the excretion of noradrenaline in the urine over 24 hours is thus a valuable aid to the diagnosis of this condition. Few clinical laboratories, however, have the facilities for noradrenaline assay, and there is a need for a simple, reliable, and fairly rapid test to detect the increased secretion of noradrenaline which is characteristic of phaeochromocytoma.

Recently it has been shown (Armstrong and McMillan, 1957; Axelrod, Senoh, and Witkop, 1958) that both adrenaline and noradrenaline are metabolized to 3-methoxy 4-hydroxy mandelic acid or vanillyl mandelic acid. Armstrong and his colleagues showed that in phaeochromocytoma the excretion of vanillyl mandelic acid was much higher than normal. They further showed that, after the tumour's removal, the excretion of vanillyl mandelic acid fell markedly, and they suggested measurement of output of vanillyl mandelic acid as a test for phaeochromocytoma.

In this paper a description is given of a relatively rapid two-dimensional paper chromatographic method for the semi-quantitative measurement of vanillyl mandelic acid in urine.

Method

Urine should be collected over a measured period of time. Although dietary control does not seem to be essential, it might be as well for the subject to avoid bananas (Waalkes, Sjoerdsma, Creveling, Weissbach, and Udenfriend, 1958), coffee, and citrus fruits, which lead to the appearance of spots which might be mistaken for vanillyl mandelic acid. The normal urinary chromatogram (Fig. 1) shows the presence of large amounts of 3-hydroxy-4-methoxyphenylhydracrylic acid, together with a smaller amount of isovanilloyl glycine; these compounds, together with traces of isovanillic and dihydroisoferulic acids, probably owe their presence to the consumption of citrus fruit containing the flavonoid hesperidin (Booth, Jones, and DeEds, 1958). The subject should also be free from undue stress during the period of collection, since many forms of stress, e.g., exercise, anaesthesia, and surgical operation, can increase the rate of secretion of both adrenaline and noradrenaline.

An ethyl acetate extract of urine (Hill, Ratcliffe, and Smith, 1959) is prepared as follows:

The urine is first acidified to *p*H 2 (indicator paper) by adding concentrated hydrochloric acid drop by drop, and a known volume (x ml.) equivalent to the amount passed in two minutes is added to ethyl acetate ($5x$ ml.) containing anhydrous sodium sulphate ($2x$ g.). The whole is vigorously shaken and placed in the refrigerator for one hour. During this period it should be shaken occasionally, the cake of sodium sulphate being broken up with a glass rod if necessary.

Chromatography of the resulting extract is carried out by some variant of the method of Armstrong, Shaw, and Wall (1956). It should be appreciated that, apart from the occasional occurrence of large quantities of some constituents, e.g., urea, hippuric acid, salicyluric acid, which may tend to lead to distorted chromatograms, successful chromatography is also dependent on atmospheric conditions owing to the volatility of solvents. Factors which favour the production of good chromatograms include the use of a non-volatile second solvent (anisole) and of Whatman No. 20 paper which gives very compact spots; successful development in the first direction may be aided by a relatively high laboratory temperature (23–25° C.), and by the choice of tanks which are small or are lined with filter-paper soaked in the solvent used (isopropanol-ammonia) to prevent excessive evaporation of solvent leading to "seizing up" at the solvent front. A certain amount of evaporation is nevertheless desirable, since this results in effectively higher R_F values. It will be evident that optimal conditions may vary from laboratory to laboratory, but the following method always gives chromatograms adequate for screening purposes.

A portion of the ethyl acetate extract (1.25x ml.) is removed and concentrated to a small volume, preferably at a low temperature. It is then transferred quantitatively to a 10 in. square of Whatman No. 20 filter paper, the size of the spot being kept as small as possible; it should be situated about 1 in. from each of two adjacent edges of the paper. Develop-

TABLE I

PHENOLIC ACIDS IN NORMAL URINE

No.	Acid	Trivial Name	Colour with Diazotized p-Nitraniline
1	4-hydroxy-3-methoxy mandelic	Vanillyl mandelic	Violet
2	3-hydroxy-4-methoxy phenyl hydracrylic	—	Blue-violet
3	4-hydroxy-3-methoxy phenyl hydracrylic	—	Violet
4	3-hydroxy-4-methoxy hippuric	Iso vanilloyl glycine	,,
5	3-hydroxy-4-methoxy benzoic	Isovanillic	,,
6	4-hydroxy-3-methoxy benzoic	Vanillic	,,
7	4-hydroxy-3-methoxy phenyl acetic	Homovanillic	Grey
8	3-hydroxy-4-methoxy phenyl proprionic	Dihydroisoferulic	Violet
9	4-hydroxy-3-methoxy phenyl proprionic	Dihydroferulic	Grey
10	p-hydroxy benzoic	—	Red
11	p-hydroxy phenyl acetic	—	Violet
12	m-hydroxy hippuric	—	Red
13	m-hydroxy phenyl hydracrylic	—	,,
14	m-hydroxy phenyl acetic	—	,,
15	m-hydroxy phenyl proprionic	—	,,
16	o-hydroxy hippuric	Salicyluric	,,
17	o-hydroxy phenyl acetic	—	Violet-red
18	5-hydroxy indole acetic	—	Red

Substances on the chromatograms which are not yet identified are represented by their colours.

ment of the chromatogram in the first direction (ascending) should proceed overnight, and the faster running direction of the paper should be used. The solvent is isopropanol—5% (w/v) ammonia (4:1). Next day the paper is dried and then run at right angles to the original direction in anisole-glacial acetic acid-water (70:27:3). (It may be necessary to add a few more drops of acetic acid in order to obtain a homogeneous mixture.) Ascending development requires five or six hours. Before running in this solvent it is advisable to "equilibrate" the paper by placing it in a closed vessel containing acetic acid vapour for 30 to 60 minutes. This procedure does not appear markedly to improve chromatograms but minimizes loss of acetic acid from the solvent, which may therefore be used several times before R_F values begin seriously to decrease.

The paper is then allowed to dry in a fume cupboard (at this stage it is impossible completely to remove anisole, which remains absorbed by the paper), sprayed lightly with 10% (w/v) anhydrous sodium carbonate and again dried before phenols are revealed by a final spray with diazotized p-nitraniline (Acheson, Paul, and Tomlinson, 1958): 5 ml. of p-nitraniline (0.2% (w/v) in N-HCl) is diluted with 50 ml. 0.1N-HCl and treated with 1 ml. of 5% (w/v) sodium nitrite. All solutions should be at 0–4° C. After two or three minutes 10 ml. of ice-cold 10% (w/v) sodium carbonate is added and the mixture used immediately. The vanillyl mandelic acid spot is readily distinguished by its position and violet colour. The acid may be estimated by visual comparison of spots (after chromatography) from 0.5, 1, and 1.5 μg. of the authentic acid with spots obtained from aliquots of the ethyl acetate extract containing quantities of vanillyl mandelic acid within this range. This refinement is scarcely necessary for the detection of phaeochromocytoma, and comparison may be made against a chromatogram derived from a mixed urine from normal subjects.

Urine specimens have been examined from four proven cases of phaeochromocytoma, from rather more than 100 other patients, most of whom were suffering from essential hypertension, and from 30 apparently normal male subjects, many of whom were investigated on several occasions.

Results

The outstanding feature of the results on the normal subjects and on patients with essential hypertension was their relative constancy, the rates of excretion of vanillyl mandelic acid lying within the range 1–2.5 μg./min. (values are not corrected for recovery; many chromatograms were larger scale ones, as recommended by Hill *et al.*, 1959). An occasional urine was encountered with a rather higher value, but in each case the patient was under some form of stress.

In contrast the patients with phaeochromocytoma excreted 7.5–11 μg./min. Visual inspection of the chromatograms was sufficient alone to distinguish these patients from the normal subjects (cf. Figs. 1 and 2).

Urine samples were examined after removal of the tumour in three cases. In two of these there was a marked fall in the excretion of vanillyl mandelic acid. In the third, both symptoms and

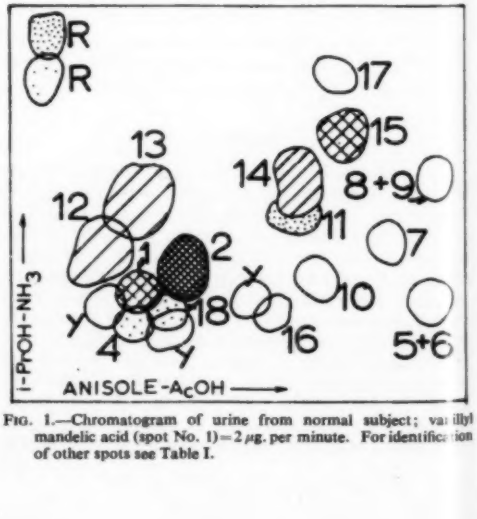

FIG. 1.—Chromatogram of urine from normal subject; vanillyl mandelic acid (spot No. 1) = 2 μg. per minute. For identification of other spots see Table I.

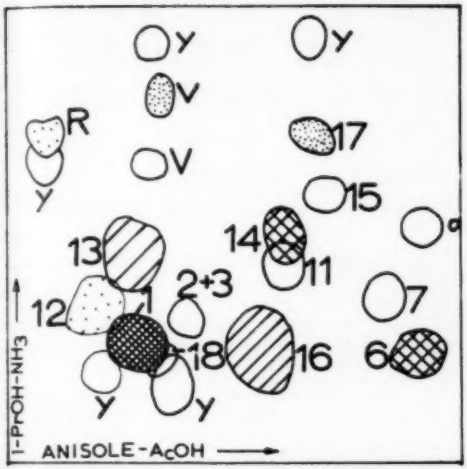

FIG. 2.—Chromatogram of urine from patient with phaeochromocytoma; vanillyl mandelic acid (spot No. 1) = 9 μg. per minute.

a high excretion of vanillyl mandelic acid persisted. The reasons for this are not as yet known.

Discussion

Biological assay of noradrenaline requires facilities for the rat or cat blood pressure techniques which few clinical laboratories possess; fluorimetric assay requires a highly sensitive and costly fluorimeter which again most clinical laboratories do not possess. In contrast, the chromatographic technique for measuring the excretion of vanillyl mandelic acid is simple and requires only apparatus which is standard equipment in the clinical laboratory.

In recent years laboratories where noradrenaline assays are made have become increasingly reluctant to accept urine specimens from other hospitals, since the great majority of specimens prove to be normal. It is suggested that examination of the urine for vanillyl mandelic acid, which can be undertaken by most clinical laboratories, should be used as a screening test. A marked increase in excretion of vanillyl mandelic acid is strong evidence in favour of the diagnosis of phaeochromocytoma. Confirmation may then be sought by submitting the specimen for noradrenaline assay.

It should be emphasized that the method recommended in this paper has largely a negative value, and that, should an apparently positive result be obtained, more detailed chromatographic investigations are essential. In addition, valuable information might be obtained if observations could be made on subjects whose food and drug intakes were known or controlled, for one at least of the above four phaeochromocytoma patients excreted unusual quantities of other substances which may be related directly or indirectly to noradrenaline metabolism. Thus excretion of vanillyl mandelic acid (10 μg./min.) was rather less than that of vanillic acid (13 μg./min., estimated in urine after acid hydrolysis). The significance of this latter has previously been discussed (Smith, 1958), and the acid may be of considerable interest, for it has been observed in large quantities not only after infusion of noradrenaline but also in adrenalectomized, amongst other, subjects. However, although the absence from these urines of large amounts of vanilloyl glycine strongly suggests that the vanillic acid was not derived from exogenous sources, such observations would clearly be of more value had they been made on patients under strict dietary control. Consumption of vanillin either as sweets, e.g., chocolate, or as medicaments, e.g., laxatives, represents an obvious danger. Also the proximity of isovanilloyl glycine to vanillyl mandelic acid on chromatograms should be noted (Fig. 1), since isovanillin may sometimes be used as a flavouring, although we have no direct evidence to this effect.

Summary

Markedly increased urinary excretions of a noradrenaline metabolite, vanillyl mandelic acid, were found in four cases of phaeochromocytoma.

A simple paper chromatographic method for detecting increased amounts of vanillyl mandelic acid in urine is described.

Results confirm the suggestion of Armstrong *et al.* (1956) that the method may be used as a screening test for phaeochromocytoma.

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THE PREPARATION AND USE OF ^{131}I-TAGGED FIBRINOGEN TO DEMONSTRATE FIBRINOLYSIS

BY

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The preparation of fibrinogen tagged with radioactive iodine (^{131}I) has been described in a preliminary communication (Clement and McNicol, 1958). The object of the present paper is to describe the chemical, electrophoretic, and chromatographic properties of the labelled protein, and to give an account of its use to demonstrate fibrinolytic activity in human plasma.

Materials and Methods

Preparation of Tagged Fibrinogen.—Radioactive iodine ($^{131}I_2$) was prepared from 1 mc. carrier-free radioactive iodide (as sodium iodide) in 0.1 ml. dilute sodium thiosulphate solution by the addition of one drop of N/$^{127}I_2$ in methanol and one drop of 0.01N H_2SO_4. Then 5 ml. of human fibrinogen in citrate saline, concentration about 4 mg. per ml., prepared by the Jaques phosphate-buffer method (Biggs and Macfarlane, 1957a), was added. The mixture was allowed to stand for five minutes at room temperature. It was then passed through a Cl-phase resin column ("amberlite" IRA 400 Cl) to remove free iodide and iodine. The tagged fibrinogen was stored in small quantities at −20° C.

Electrophoresis.—Electrophoresis was carried out in a horizontal electrophoresis tank on Whatman No. 1 paper for 16 hours at 130 volts and 1 ma. for each 5 cm. paper. Strips were stained for protein with bromophenol blue.

Chromatography.—This process was carried out by ascending chromatography in butanol acetic acid solvent. The strips were stained for amino-acids with diazotized sulphanilic acid and 10% potassium carbonate, and with 1% palladium chloride for iodide. Monoiodotyrosine, diiodotyrosine, and potassium iodide were run as markers.

Fibrin Estimation.—The method employed is a colorimetric one which consists in measuring the optical density on the addition of Folin Ciocalteau phenol reagent to an alkaline solution of fibrin (Holburn, 1951). The optical density of the colour developed is directly proportional to the concentration of tyrosine. This was measured by comparison with a standard tyrosine solution in a photoelectric spectrophotometer, the results being read off a calibration curve, and converted to milligrammes of fibrin by means of a conversion factor.

Thrombin.—Thrombin was prepared from expired bank blood by the method of Biggs and Macfarlane (1957b), and was stored at −20° C. Fresh samples were used for each experiment and were diluted with saline to give a clotting time of 10 seconds with an equal volume of plasma.

Buffer.—To 10 ml. of stock solution of sodium acetate/sodium barbitone (9.714 g. of Na acetate and 14.714 g. Na barbitone made up to 500 ml. with distilled water) was added 10 ml. of 0.1N HCl and 30 ml. distilled water. Fresh buffer was made up for each experiment.

Glassware.—Glassware used in the production of the tagged fibrinogen was acid washed, alkali washed, thoroughly rinsed with distilled water, and dried in a hot air oven; the importance of clean glassware cannot be overemphasized. Other glassware was washed in a solution of "deosan" detergent, thoroughly rinsed, and dried overnight in a hot air oven at 100° C. In some later experiments glassware was sterilized by heating to 160° C. for one hour in a hot air oven.

Steps were taken at all stages to prevent bacterial contamination, and random cultures were sterile.

Chemical Properties of Tagged Fibrinogen

Efficient tagging depends on scrupulous cleanliness of glassware. If glassware is not clean, only about 70% of the total radioactivity may be in the clot formed by the addition of thrombin. The results to be described were all obtained with fibrinogen in which 88–94% of the radioactivity was coagulable.

As previously reported (Clement and McNicol, 1958), when 10% trichloracetic acid was added to precipitate the proteins, 98% of the activity was protein bound and came down with the precipitate.

Dilute sulphuric acid and 0.01N potassium dichromate were added to the tagged fibrinogen to change any free iodide to iodine by an oxidation

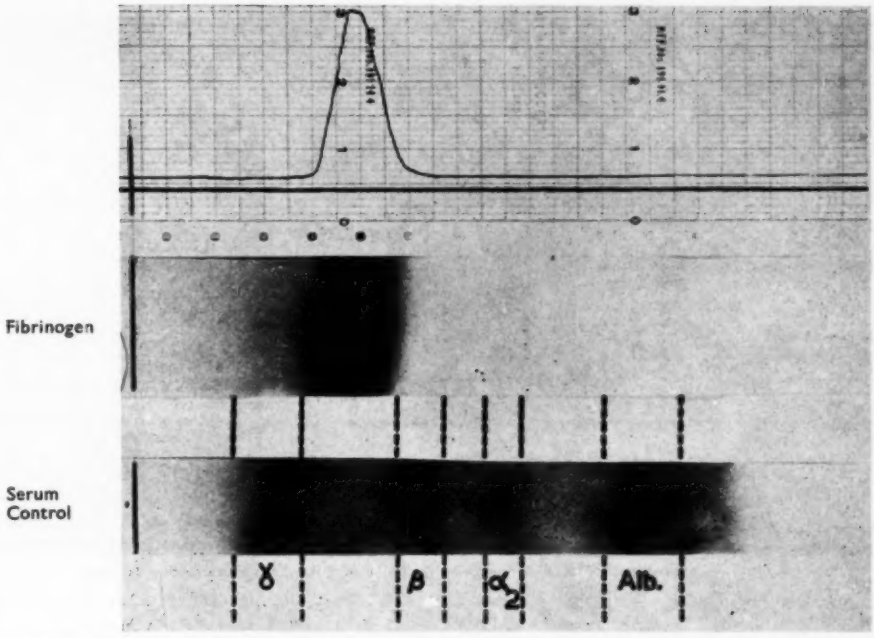

FIG. 1.—Electrophoresis of fibrinogen tagged with radioactive iodine. A control strip of normal serum run simultaneously is shown below, and a recording of the radioactivity in the fibrinogen above the fibrinogen strip.

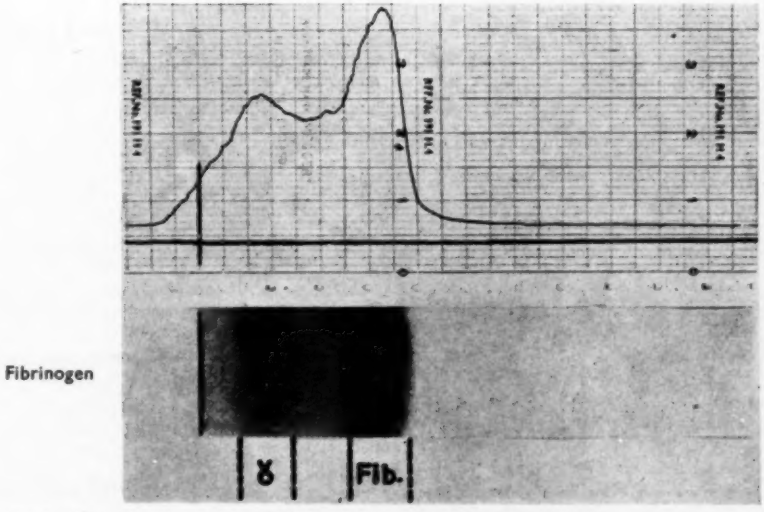

FIG. 2.—Electrophoresis strip of tagged fibrinogen contaminated with tagged γ globulin. A recording of the radioactivity is shown above the electrophoresis strip

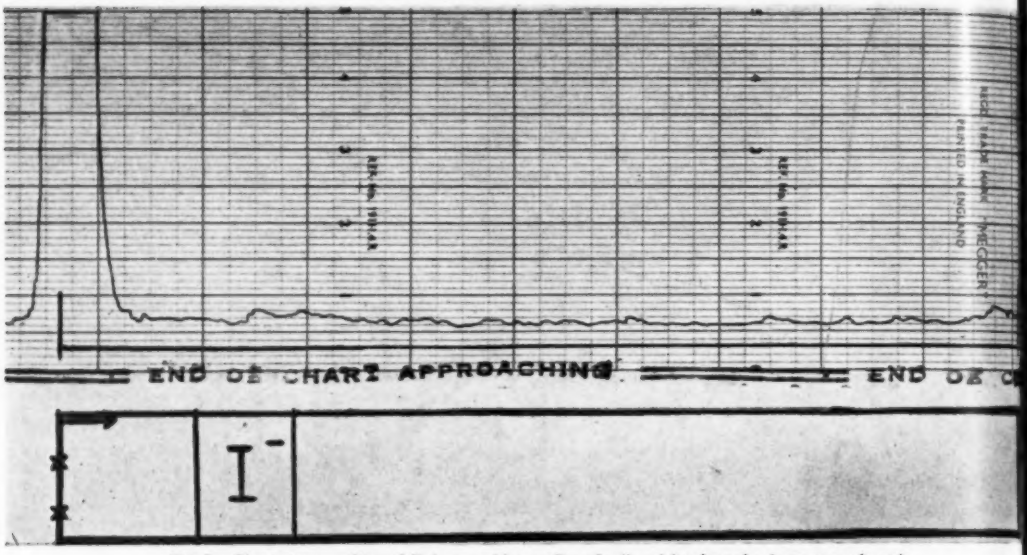

FIG. 3.—Chromatogram of tagged fibrinogen with recording of radioactivity above the chromatography strip.

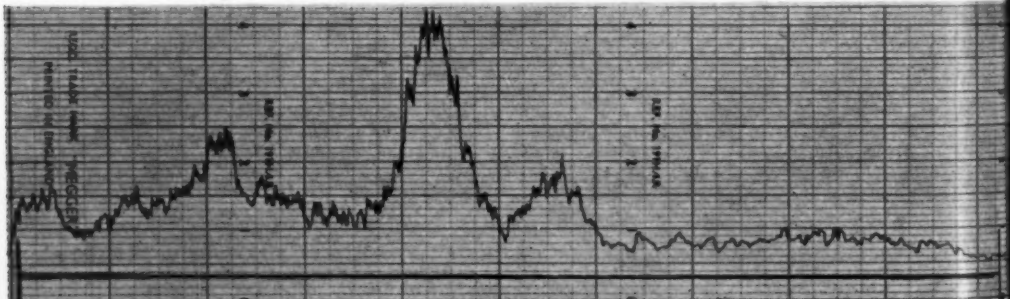

FIG. 4.—Chromatogram of tagged fibrinogen after hydrolysis with trypsin. A recording of the radioactivity is shown above the chromatography strip.

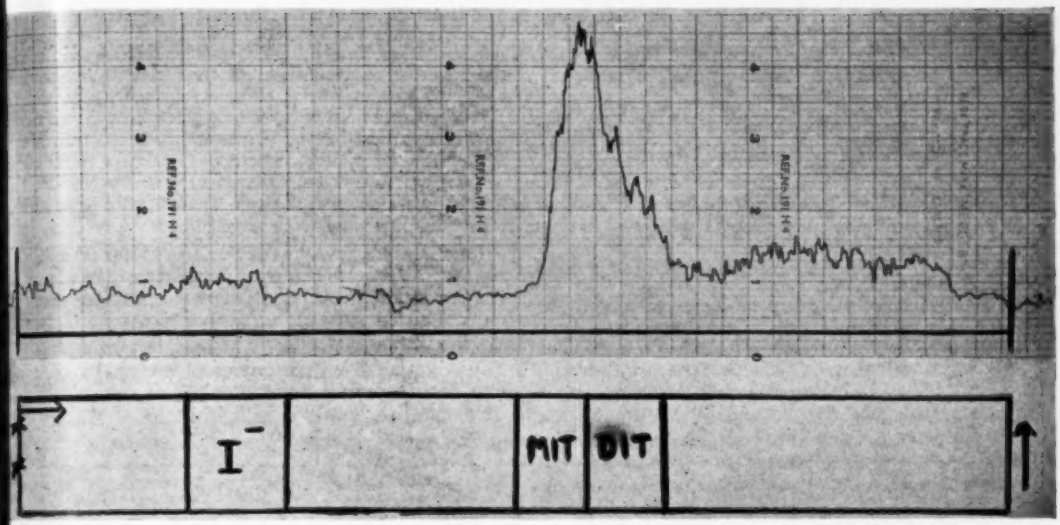

FIG. 5.—Chromatogram of tagged fibrinogen after hydrolysis with 6N HCl. A recording of the radioactivity is shown above the chromatography strip.

reaction. Chloroform extraction to remove free iodine was then carried out, and only 4% of the radioactivity was extractable.

Electrophoresis and Chromatography of the Tagged Fibrinogen

Fig. 1 shows an electrophoresis strip of the tagged fibrinogen with a control strip of normal serum run simultaneously. The strip was scanned for radioactivity by passing it between two Geiger-Müller tubes linked through a ratemeter to a recording milliammeter. All the radioactivity is seen to be with the fibrinogen. Electrophoresis should be carried out with each batch of fibrinogen to exclude contamination with other proteins which would also tag. For instance, in Fig. 2 tagged globulin can be seen to be contaminating this particular batch of fibrinogen.

Fig. 3 shows a chromatogram of the tagged fibrinogen scanned for radioactivity which is all at the origin: no iodide was washed out. Chromatography was repeated after trypsin hydrolysis. Fig. 4 shows radioactivity, 63% of which is opposite mono- and di-iodotyrosine markers. Chromatography after hydrolysis with 6N HCl shows radioactivity which is opposite mono- and di-iodotyrosine markers (Fig. 5).

The electrophoresis strip demonstrates that the iodination procedure described in our previous communication (Clement and McNicol, 1958) does in fact produce a protein, to which radioactive iodine is attached, with the electrophoretic mobility of fibrinogen. Chromatography of the untreated fibrinogen confirms that all the iodine is bound to the fibrinogen. Chromatography after trypsin hydrolysis suggests that much of the binding is as mono- and di-iodotyrosine. That tagging to tyrosines has not taken place after hydrolysis can be assumed from the result of chromatography after hydrolysis with 6N HCl. It will be seen that most of the radioactivity is with mono- and di-iodotyrosine. The *p*H produced by 6N HCl is most unfavourable to tagging, which has almost certainly taken place before the HCl was added.

Use of the Tagged Fibrinogen to Demonstrate Fibrinolytic Activity in Plasma

Principle.—The tagged fibrinogen and plasma, in varying dilutions, were clotted with thrombin and incubated together for varying times. Fibrinolytic activity was measured by the reduced proportion of the total radioactivity which could be removed with the clots.

Technique.—Ten healthy subjects, aged between 20 and 40 years, were used. Blood was withdrawn by clean venepuncture at the same time of day, and was immediately mixed in an ice-cold container with 1 part of 3.8% sodium citrate B.P. to 9 parts of blood. Platelet-free plasma was obtained by high-speed centrifugation at 4° C. To 20 ml. of the plasma 0.1 ml. of tagged fibrinogen was added and thoroughly

TABLE I
QUANTITIES OF TEST PLASMA AND SALINE REQUIRED FOR INCREASING PLASMA DILUTIONS

	Tube No.					
	I	II	III	IV	V	VI
Plasma (ml.) ..	3	2	1	0·5	0·2	0·1
Saline ,, ..	1	2	3	3·5	3·8	3·9
Thrombin ,, ..	1	1	1	1	1	1
Plasma concentration (%) ..	60	40	20	10	4	2

mixed. The experiment was then set up as quickly as possible at 37° C.

Table I shows the design of the experiment. For each subject, three sets of six test-tubes were set up, with progressive plasma, and therefore iodinated fibrinogen, dilutions. Each tube was clotted with 1 ml. thrombin to give the final plasma dilutions shown. From the first set of six tubes the clots were removed immediately; the second and third sets of six tubes were incubated for four and 22 hours respectively, when the clots were removed. For all 18 tubes the radioactivity in the clots, and in the fluid from which they had been removed, was counted in a well-type scintillation counter.

For each subject the experiment was repeated on a subsequent day, collection of specimens and setting-up of the experiment being carried out at 37° C.

Results.—These are set out in Table II and Fig. 6. Using unincubated tubes as a base-line, percentage lysis was calculated from the increased proportion of the total radioactivity remaining in the fluid from which the clot had been removed. For example, if the percentage of the total radio-

TABLE II
PERCENTAGE LYSIS AFTER FOUR AND 22 HOURS' INCUBATION WITH "SETTING UP" AT 4° C. AND 37° C.*

Plasma Concentration (%):		60	40	20	10	4	2
4° C.	4 hr. incubation	2·34 (0–4·7)	3·74 (0·8–6·2)	5·65 (1·2–11·9)	7·06 (1·6–15·7)	15·3 (6·0–32·8)	50·8 (12·2–100)
	22 hr. incubation	30·76 (1·1–98·5)	38·93 (3·3–100)	58·24 (10·5–100)	76·37 (43·0–100)	99·55 (97·5–100)	100
37° C.	4 hr. incubation	2·03 (0–5·7)	2·67 (0·3–6·4)	3·63 (1·4–7·0)	4·39 (0·9–9·1)	7·55 (2·2–15·9)	19·8 (2·1–68·3)
	22 hr. incubation	10·43 (0·3–38·8)	16·92 (0·3–86·0)	22·81 (2·4–96·5)	30·42 (6·6–100)	65·71 (6·7–100)	85·72 (18·2–100)

* Mean figures for the 10 subjects are shown, with ranges below in brackets.

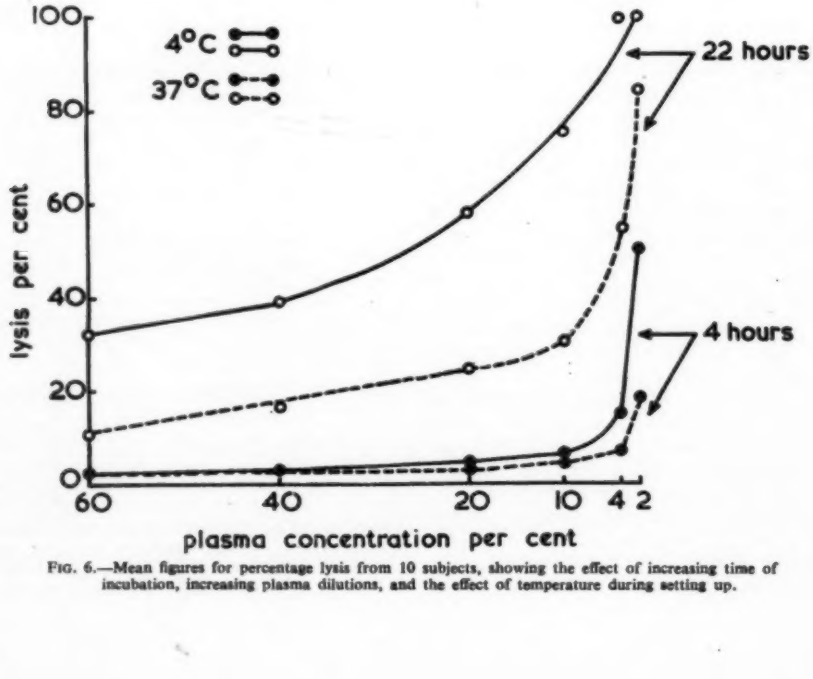

FIG. 6.—Mean figures for percentage lysis from 10 subjects, showing the effect of increasing time of incubation, increasing plasma dilutions, and the effect of temperature during setting up.

TABLE III

MEASUREMENT OF FIBRINOLYTIC ACTIVITY USING TAGGED FIBRINOGEN COMPARED WITH A TYROSINE ESTIMATION*

Duration of Incubation (Hours):	1	2	3	4	5	6	24
Subject 1	1·3	1·8	2·7	3·8	3·4	8·9	100
	5·1	0·7	4·8	1·7	6·9	17·6	100
,, 2	0·6	1·3	2·5	3·9	14·7	23·1	100
	2·3	7·5	3·1	11·9	18·2	29·6	100
,, 3	0·7	0·9	1·3	3·6	4·3	16·3	100
	1·8	3·2	5·1	8·4	6·8	25·1	100
,, 4	2·6	1·2	No readings	3·7	4·8	7·1	100
	4·9	6·3	made	5·7	7·8	16·6	100
,, 5	0·7	3·4	10·6	21·5	28·6	41·3	100
	0	6·8	16·2	28·8	39·2	62·7	100
,, 6	4·5	8·9	17·4	55·2	71·6	100	100
	9·4	4·7	27·3	65·7	88·4	100	100

* The figures show fibrinolysis per cent. for six subjects for varying periods of incubation. The upper of each pair of results was obtained using tagged fibrinogen, the lower using the tyrosine estimation method.

activity left in the fluid after removal of the clot was before incubation x%, and after incubation y%, percentage lysis is calculated as $\frac{(y-x)}{100-x} \times 100$.

Mean figures, and ranges, for percentage lysis for the 10 subjects, for varying dilutions, incubation periods, and temperatures during setting up, are shown in Fig. 6 and Table II.

Assessment of Fibrinolytic Activity with Tagged Fibrinogen Compared with a Tyrosine Estimation

Technique.—Platelet-free plasma was obtained by the methods described above from six subjects, of whom four (subjects 1–4) were healthy young adult volunteers, and two (subjects 5 and 6) were patients with established cirrhosis of the liver.

The plasma from each patient was divided into two equal portions, to one of which was added 0.1 ml. of tagged fibrinogen. Two sets of eight test-tubes were prepared, each tube containing 2 ml. saline and 2 ml. buffer solution. To each of the first set of eight tubes was added 0.5 ml. plasma, and to each of the second set of eight tubes 0.5 ml. plasma with tagged fibrinogen. All 16 tubes were then clotted with 0.3 ml. thrombin. From one tube in each series the clot was removed after five minutes. The remaining 14 tubes were incubated at 37° C.; clots were removed from successive tubes in both series at hourly intervals for six hours, and at 24 hours.

For the radioactive series of tubes, the radioactivity in the clots, and in the fluid from which they had been removed, was measured and percentage lysis calculated.

The clots were removed from the non-radioactive tubes on glass rods. The clots were washed twice in normal saline, and the fibrin content of each clot was determined. Percentage lysis for each tube was calculated from the diminished content of fibrin present compared with that in the unincubated tube.

Results.—The results obtained with both methods for the six subjects are set out in Table III, and show that there is fair general agreement between the assessment of fibrinolytic activity using tagged fibrinogen and using a conventional method involving tyrosine estimation. The results with the tagged fibrinogen are on the whole somewhat lower than those obtained by the estimation of tyrosine; however, the number of observations is too small to justify statistical analysis.

Discussion

The method of tagging fibrinogen with radioactive iodine which has been described in this paper is easily and quickly carried out. If scrupulously clean glassware is used about 90% of the radioactivity in most batches is in the clot produced by the addition of thrombin. This is a significant improvement on the method of Shulman and Tagnon (1950) in which only about 75% of the radioactivity was coagulable. Another relatively much more complex and lengthy method of tagging fibrinogen with radioactive iodine has also been described by Ambrus, Back, Mihalyi, and Ambrus (1956), who do not, however, state how much of the radioactivity in the final solution is coagulable.

Use of the Tagged Fibrinogen to Demonstrate Fibrinolysis.—The results obtained in these experiments are broadly similar to those obtained using a conventional technique by Fearnley and Lackner (1955). Lysis is much more marked after incubation for 22 hours than after incubation for four hours. Study of the figures shows that the differences between the results for the two periods of incubation are highly significant, despite the wide scatter of results in the two experiments. (In a t test, for the most concentrated tubes, $P<0.05$; for all others, $P<0.01$.)

The results also confirm Fearnley and Lackner's observation that fibrinolytic activity is relatively much stronger in dilute solutions.

It will be seen that fibrinolytic activity is greatly reduced in the experiments set up at 37° C. compared with those set up at 4° C.: the reduction in activity is highly significant after incubation for 22 hours (in a t test, for the most concentrated tubes, $P<0.05$; for all other tubes, $P<0.01$), but the reduction in activity is not statistically significant after only four hours' incubation. This evidence of the thermolability of the enzymes is in keeping with the findings of Fearnley and Tweed (1953).

It will be seen that in the two patients with cirrhosis of the liver fibrinolytic activity is more active than in the normal subjects: this finding is in keeping with the work of Kwaan, McFadzean, and Cook (1956).

The use of tagged fibrinogen to measure fibrinolytic activity should be of value in situations where other methods are unsuitable because of technical difficulties, for example, excessive turbidity preventing the use of the photospectrometer. Also, survival studies with tagged fibrinogen *in vivo* may offer a more physiological approach to certain problems of fibrinolysis.

Summary

A method of preparing fibrinogen tagged with radioactive iodine (^{131}I) has been described. The chemical, electrophoretic, and chromatographic properties of the fibrinogen have been demonstrated. The tagged fibrinogen has been used to demonstrate fibrinolytic activity in human plasma, and to confirm that fibrinolysis is relatively more active in dilute solutions, and that fibrinolytic activity, studied *in vitro* in normal subjects, is thermolabile. The results obtained using tagged fibrinogen to measure fibrinolytic activity are similar to those obtained from a conventional method involving tyrosine estimation.

We thank Professor L. J. Davis, Dr. E. M. McGirr, and Dr. A. S. Douglas for their guidance in this work and the preparation of this paper, Dr. W. C. Watson, who carried out the electrophoresis, Miss K. Mair, who prepared the fibrinogen and thrombin, and Dr. A. I. Macdougall, who advised on certain aspects of the preparation of fibrinogen. We are also grateful to the Advisory Committee on Medical Research for Scotland, and to the Rankin Fund, for defraying part of the cost of the equipment, and to the Medical Research Council for technical assistance.

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CHRISTMAS FACTOR (IX) DEFECT IN A CASE OF HAEMORRHAGIC THROMBOCYTHAEMIA

BY

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The mechanism of bleeding in haemorrhagic thrombocythaemia is obscure. Although by definition there is always a raised platelet count, the haemorrhagic episodes cannot be satisfactorily correlated with the level of the count. In fact, thrombocythaemia can exist without bleeding manifestations (Fanger, Cella, and Litchman, 1954). The most commonly occurring laboratory defect is an abnormal bleeding time, this being increased in approximately 50% of cases (Hardisty and Wolff, 1955). The introduction of the thromboplastin generation test by Biggs and Douglas in 1953 provided a valuable method of investigation, and has been applied in many of the reported cases of haemorrhagic thrombocythaemia. Attention has, however, been focused mainly on the platelets, and the results have varied considerably. This paper presents details of a case of haemorrhagic thrombocythaemia showing a Christmas factor deficiency.

Methods

Blood Counts.—In this laboratory three were performed as described by Dacie (1950).

Clotting Mechanism.—Tests of clotting were as described by Biggs and MacFarlane (1957).

In the one-stage prothrombin test (Quick) a saline emulsion prepared from a stock of acetone-dried human brain powder, selected to give a normal clotting time of 12 ± 1 sec., was used.

The thromboplastin generation tests were based on the method of Biggs and Douglas (1953), the collection of blood being in accordance with Biggs and MacFarlane (1957). The only modification was that clotting was observed on slides on a specially constructed thermostatically-controlled warm stage, which enabled the moment of clotting to be gauged accurately.

Absorption by $Al(OH)_3$.—Platelet-rich normal plasma was centrifuged and the supernatant removed so as to increase the platelet concentration 10 times. The platelets were resuspended and the plasma incubated for three hours. After re-centrifuging, the supernatant was removed and treated with $Al(OH)_3$. This plasma and the same plasma before platelet concentration were compared against known Christmas disease serum in the thromboplastin generation tests. There was no significant difference in the curves produced.

Case History

Miss G.H., aged 61, presented in 1946 with a haematemesis from a peptic ulcer which later perforated. At operation the spleen was thought to be enlarged. In June, 1948, she complained of persistent headaches.

On examination, the spleen was just palpable and the blood count showed: Hb 94% (31.9 g./100 ml.); R.B.C.s 5,850,000 per c.mm.; W.B.C.s 28,400 per c.mm. (neutrophils 64.5%, juveniles 12.5%, eosinophils 5.5%, basophils 3.0%, myelocytes 6.5%, monocytes 1.0%, lymphocytes 7.0%). There was no comment on the platelets.

She was considered to be a case of chronic myeloid leukaemia and was kept under observation without treatment. The blood picture persisted for the next few years with little variation in the differential pattern.

In 1951 she was admitted to this hospital for blood transfusion following a haematemesis. A platelet count, recorded for the first time, was 1,000,000 per c.mm. Over the following years spontaneous haemorrhagic manifestations with ecchymoses became more frequent, and by 1955 the platelet count had risen to 2,000,000 per c.mm. and the splenomegaly had become more marked. The sternal marrow was investigated. This showed an increase of megakaryocytes and was not typical of leukaemia. A diagnosis of haemorrhagic thrombocythaemia was substituted for that of chronic myeloid leukaemia. The Lee and White clotting time was 6 min. 30 sec., the clot retraction was normal, and the bleeding time was 5 min. 10 sec.

In December, 1957, she was again admitted for transfusion following haematemesis. During the next 10 months the platelet count ranged from 835,000 per c.mm. to 2,000,000 per c.mm. The bleeding time never exceeded 5 min. 15 sec. and the Quick one-stage prothrombin index varied between 65% and 100%. A prothrombin consumption test carried out in October, 1958, before treatment, gave a normal

value of 13% and a thrombin generation test at the same time showed no abnormality.

In view of the Leeds experience (Fountain, 1958), treatment with radioactive phosphorus was suggested, and on October 31, 1958, the patient received 5 mc. ^{32}P orally. By November 25 the white count had fallen to 5,600 per c.mm. and the platelet count to 500,000 per c.mm. By January, 1959, it had reached 260,000 per c.mm.

Thromboplastin Generation Studies.—The first test (Fig. 1) was performed in November, 1956. Using a normal platelet suspension, no abnormality was observed; but when the patient's platelets were used there appeared to be a mild serum defect which was less apparent when her own plasma was used with her serum. At this stage it was impossible to exclude chance variation within the normal range.

We were unable to repeat the tests until July, 1958 (Fig. 2). The platelet count was then 2,000,000 per c.mm. and the prothrombin index 100%. The tests were carried out using, respectively, pooled normal platelet suspension, brain extract platelet substitute (Bell and Alton, 1954), and the patient's own platelets, unstandardized and standardized by dilution to conform to a normal peripheral platelet count.

In each test the same phenomena were observed, but the serum defect had become more obvious. This defect was partially corrected by the patient's $Al(OH)_3$-treated plasma, which itself behaved normally when tested with normal serum. In contrast with the more usual findings in Christmas disease, the curves reached normal levels after four to seven minutes' incubation. It is of interest to note (Fig. 2) that this defect is at least as obvious when using platelet substitute as when using suspensions of either normal or patient's platelets.

A further series of tests was performed in November, 1958, on blood collected in October, before treatment with ^{32}P. They were carried out using platelet substitute and patient's standardized platelets respectively. The patient's plasma and serum had been stored at −20° C. for three weeks.

Similar results were again noted and the correcting ability of the plasma was still present (Fig. 3). Two weeks later, however, using the same plasma and serum, although the serum defect remained, this had now disappeared (Fig. 4*a*). In order to confirm that this was in fact a Christmas factor deficiency the serum was tested against two known Christmas disease sera, one kindly supplied by Dr. F. G. Bolton (Fig. 4*b*). No mutual correction could be demonstrated. Owing to the shortage of plasma and the difficulty in contacting the patient, we were most unfortunately unable to test her plasma against known Christmas-deficient serum.

In November, 1958, three and a half weeks after the administration of ^{32}P, when the platelet count had fallen to 500,000 per c.mm., blood was again collected and tested (Fig. 5). There was now no abnormality in thromboplastin generation. Since traces of thromboplastin in the plasma could have given an apparent correction of the patient's original serum defect in the thromboplastin generation test the possibility arose that during the preparation of this plasma for the test, excess of platelets might have produced so much that some remained

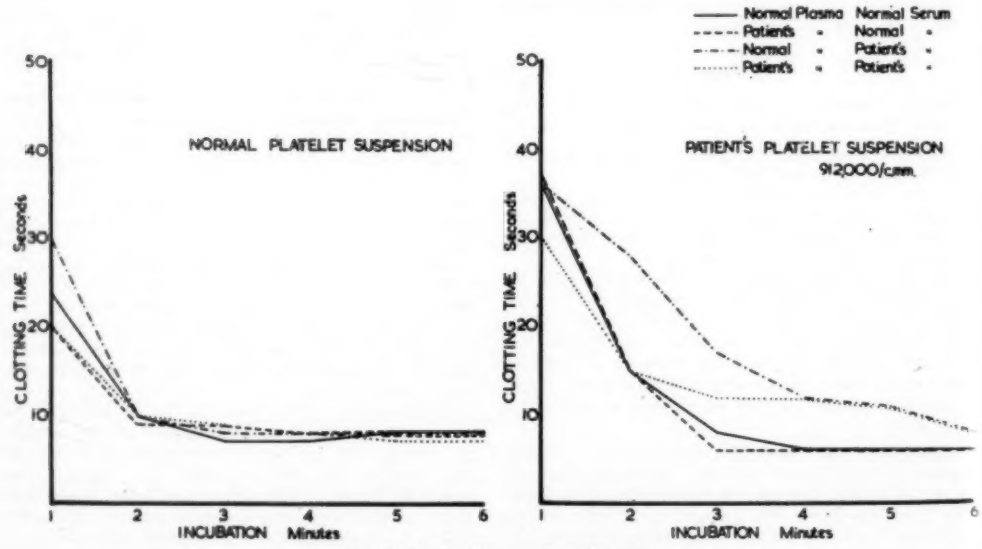

FIG. 1.—Results of tests performed in 1956.

unabsorbed, in spite of the recognized avidity of the $Al(OH)_3$ for it. The limited experiment described above was devised to exclude this.

Dr. Bolton kindly carried out assays of Christmas factor in these sera. Before treatment the level was 30% ; three and a half weeks after treatment it had risen to 55%, and two months later it was 137%. The lower limit of normal for this method is 68% (Bolton and Clarke, 1959).

Discussion

Using the thromboplastin generation test, Hardisty and Wolff (1955) in five, and Shaw and Oliver (1958) in six, cases of thrombocythaemia found evidence of platelet dysfunction. Soulier, Alagille, and Labrieu (1957) were unable to find any consistent abnormality amongst 27 cases, and Fountain (1958) in two found no abnormality at

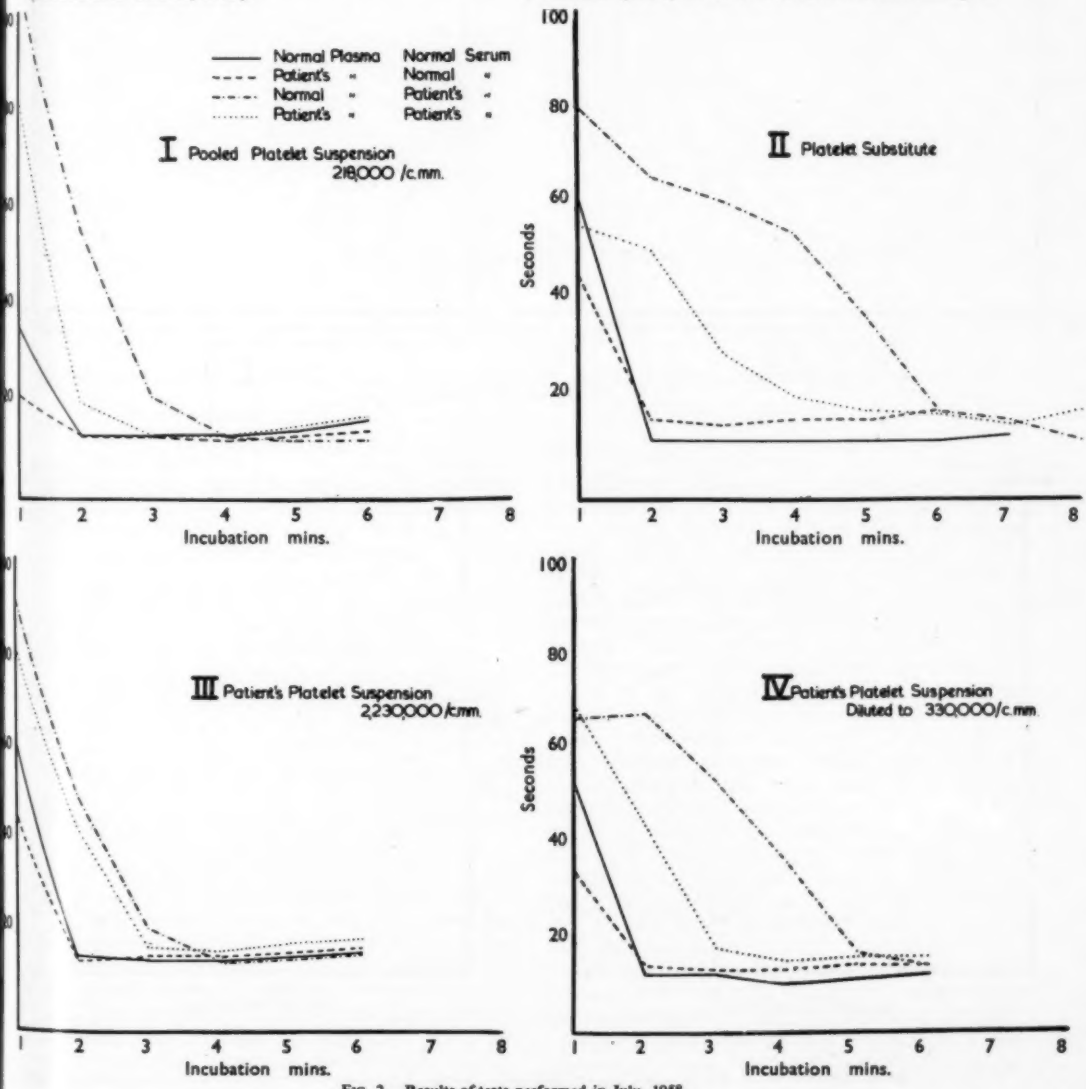
Normal Plasma Normal Serum
Patient's " Normal "
Normal " Patient's "
Patient's " Patient's "
I Pooled Platelet Suspension 218,000 /c.mm.
II Platelet Substitute
III Patient's Platelet Suspension 2,230,000 /c.mm.
IV Patient's Platelet Suspension Diluted to 330,000/c.mm.
Seconds
Incubation mins.

FIG. 2.—Results of tests performed in July, 1958.

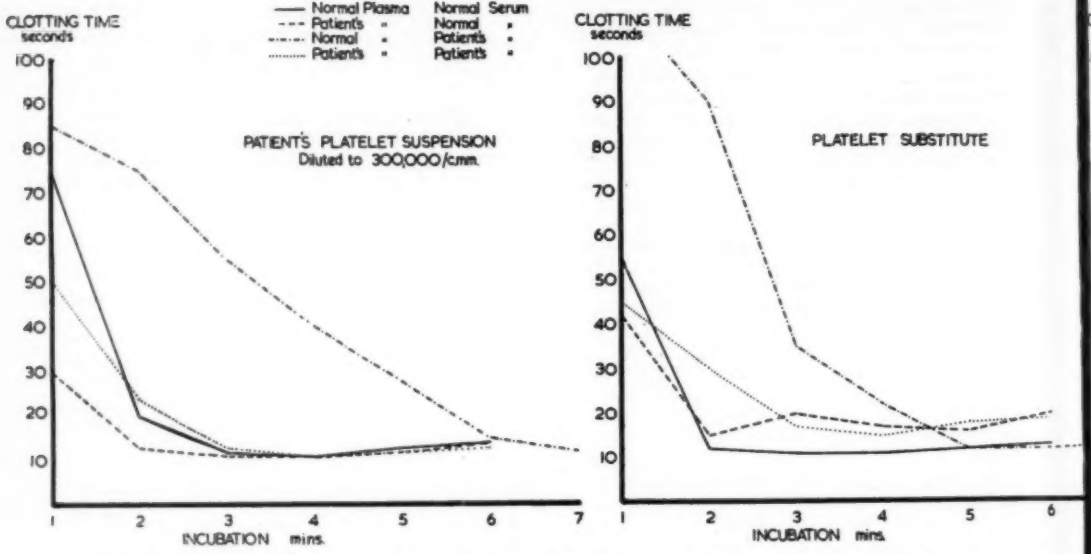

FIG. 3.—Tests performed on blood collected in October, 1958, before treatment with ^{32}P. (Plasma and serum had been stored for three weeks before testing.)

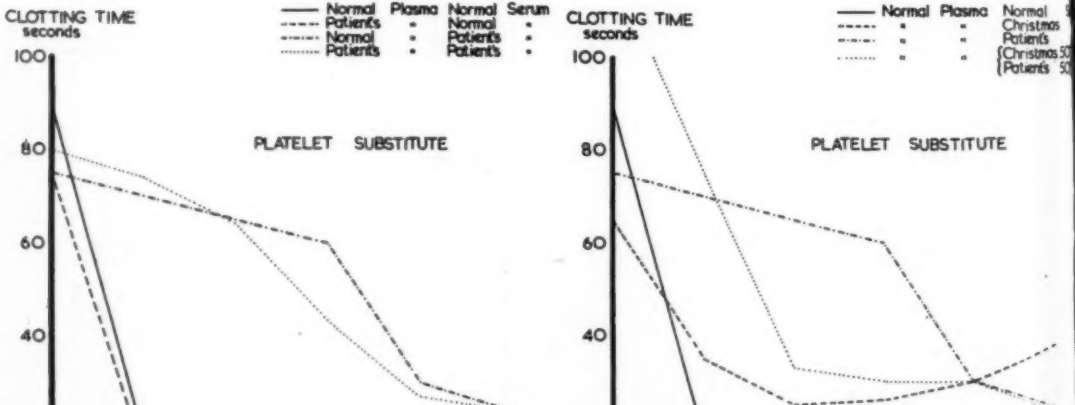

FIG. 4.—(*a*) Same specimens as in Fig. 3, after further two weeks' storage. (*b*) Graph showing lack of mutual correction with Christmas disease serum.

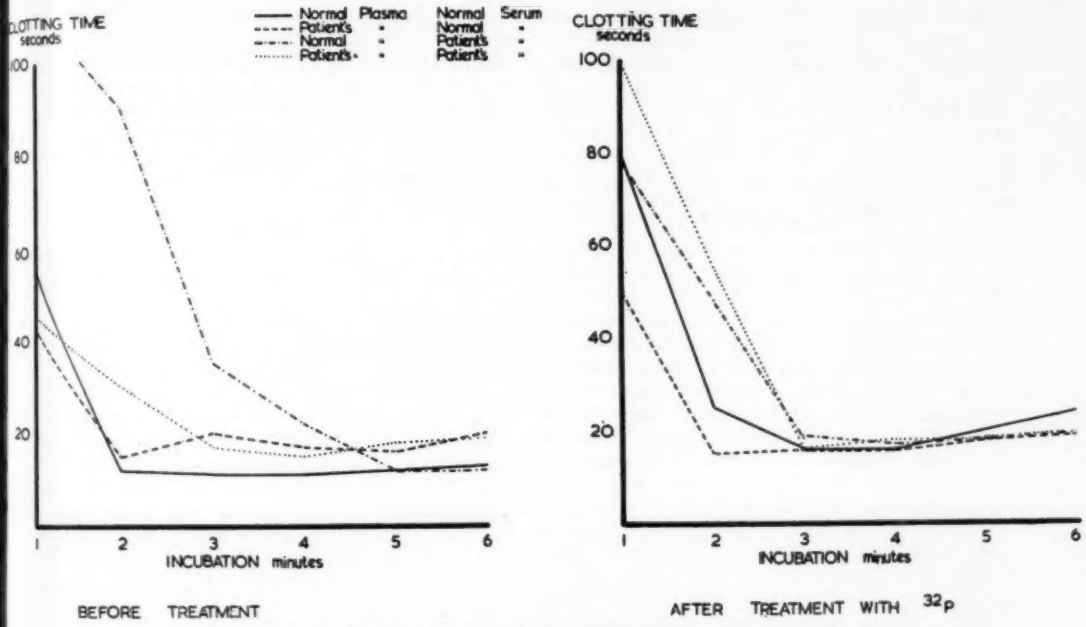

FIG. 5.—Comparison of results of tests before (October, 1958) and after (November, 1958) treatment.

all. Of these, only Fountain tested the patient's plasma and serum. It is of interest that Bonnin (1956) and Perry and Craddock (1958) were able to restore normal function to defective platelets by incubating them in normal plasma and serum, although their cases were not thrombocythaemic. There is thus a wide range of results, but the emphasis has been on platelet function.

In our case a serum defect has been observed and, although tests have been performed on one occasion only since the platelet count has fallen, the fact that it has now disappeared points to a link between it and the thrombocythaemia. This defect appears to be identical with Christmas disease, in so far as we have been able to establish it against known Christmas disease sera.

The key seems to lie in the ability of this patient's plasma to correct her own serum defect. It is not unreasonable, therefore, to assume that the total Christmas factor in her blood is not appreciably diminished and that, during the process of clotting, some only of it is liberated into the serum. Christmas factor is readily absorbed from plasma by $Al(OH)_3$. Since the patient's plasma after such treatment is able to correct the serum defect, in contradistinction to normal plasma so treated, it could indicate that the Christmas factor in the patient's plasma differs from that in normal plasma, in that it is not so readily absorbed. In view of Bergsagel's work (1955), in which it is suggested that Christmas factor exists normally in an inactive form, becoming activated in the presence of calcium and A.H.G. (VIII) in the initial stages of clotting, this difference may merely mean that the Christmas factor in the patient's plasma has only been partially activated and that the inactivated fraction is resistant to absorption; it then undergoes complete activation during the thromboplastin generation test, and is thus able to replace the deficiency of Christmas factor in the patient's test serum. The fact that after storage her plasma no longer corrects her serum defect (Fig. 4a) is consistent with a gradual change of this fraction into a form readily absorbed by $Al(OH)_3$. It may also be that only the activated fraction can be liberated into the serum during clotting, which would explain the apparent deficiency in the patient's serum. It would not, however, exclude the possibility that Christmas factor is partially consumed during the process of clotting in the presence of excess platelets (Biggs, 1959, personal communication).

If this explanation is acceptable, then, in order to explain any association with thrombocythaemia, it becomes possible to postulate that platelets also play a part in the activation of Christmas factor and that any platelet abnormality may interfere with this mechanism. Shaw and Oliver (1958) favour a qualitative abnormality, suggesting that platelets, owing to their rapid formation, may be deficient in some essential factors. Hardisty and Wolff (1955), in fact, demonstrated low platelet serotonin levels in all their five cases. Spaet, Bauer, and Melamed (1956), on the other hand, find it difficult to believe that the excess of platelets in thrombocythaemia is not more than enough to compensate for any mild qualitative defect, and put forward the view that the fundamental defect is a coagulation disorder resulting from the anticoagulant effect of an excess of normal platelets. They quote Tocantins and Carroll (1949) and Klein and Farber (personal communication) in support. Shaw and Oliver also suggest this as an alternative.

In our case, such an explanation does not in itself account for the correcting phenomenon, but it does lend support to one based on a quantitative defect. Our findings may merely mean that a sufficient increase in the number of platelets alone can throw the activating mechanism out of balance, resulting in an apparent but not real deficiency of Christmas factor. On the other hand it is possible that an excess of A.H.G. may also do this and, in the thromboplastin generation test itself, mask rather than correct by replacement a Christmas deficiency in the serum (Biggs, 1959, personal communication). O'Brien (1959) has recently suggested that heparin inhibits the activation of Christmas factor in the earliest stages of clotting. We have been unable to assess either mechanism in our case.

It is difficult to be dogmatic when one considers the variety of apparent defects found in association with thrombocythaemia, and also the complexity of the coagulation mechanism itself. But explanations based on optimum concentrations do allow of a range of reactions when the equilibrium is disturbed. Nevertheless, this Christmas factor defect, if it represents a true Christmas disease found coincidently, is a curious one in that it was most in evidence when the platelets were high, started to disappear as they fell in response to ^{32}P, and ceased to be demonstrable when they had reached a normal level. The argument that the defect in our case is intimately associated with the thrombocythaemia, as part of the syndrome of haemorrhagic thrombocythaemia, is difficult to resist.

Summary

A case of haemorrhagic thrombocythaemia is described having a Christmas factor (Factor IX) serum deficiency which is corrected in the thromboplastin generation test by the patient's $Al(OH)_3$-treated plasma. After treatment with ^{32}P this deficiency as shown in the thromboplastin generation test disappeared, and the Christmas factor assay, previously low, returned to normal.

It is suggested: (*a*) that in the initial stages of clotting an optimum concentration of platelets may be necessary for the full activation of Christmas factor.

(*b*) That if this is so, the deficiency of Christmas factor in the serum may be due to retention or consumption of the inactivated fraction during clotting.

We wish to thank Dr. J. M. Naish and Dr. Alistair MacLeod for their kindness in allowing us to investigate and report this case, Mr. G. Crane for his considerable assistance, Dr. F. G. Bolton for kindly assaying the Christmas factor for us, and Dr. Rosemary Biggs for her invaluable criticism in preparing this paper. Last but not least we would like to thank the patient for her invariable patience and her intense interest in everything we were doing.

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J. clin. Path. (1959), **12**, 557.

ON THE DIFFERENTIAL DIAGNOSIS OF THROMBOTIC MICROANGIOPATHY IN ENDOMETRIAL CURETTINGS

BY

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(RECEIVED FOR PUBLICATION MAY 9, 1959)

In a paper published three years ago on the histological diagnosis of thrombotic purpura during life I reported three cases in which the characteristic thrombotic microangiopathy was present in biopsy material (Symmers, 1956). In one of the cases the vascular lesions were found in the basal stratum of the endometrium and in endocervical fragments in a specimen obtained by curettage: the operation had been performed because of severe menorrhagia at the onset of the illness, when there were no clinical grounds for suggesting the diagnosis of thrombotic purpura. The patient's satisfactory condition after the curettage and the absence of diagnostic haemato-

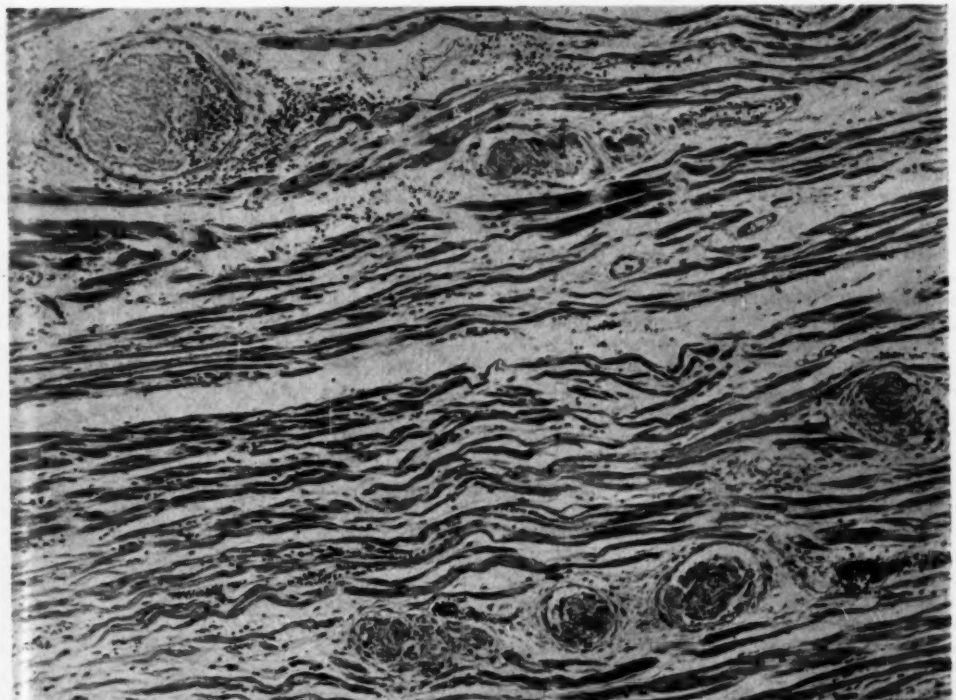

FIG. 1.—Characteristic field of myocardium in a case of thrombotic purpura, showing thrombotic microangiopathy affecting several vessels, which are much dilated and contain granular thrombi. (The black dots in the photograph are erythrocytes.) (Haematoxylin-eosin.)

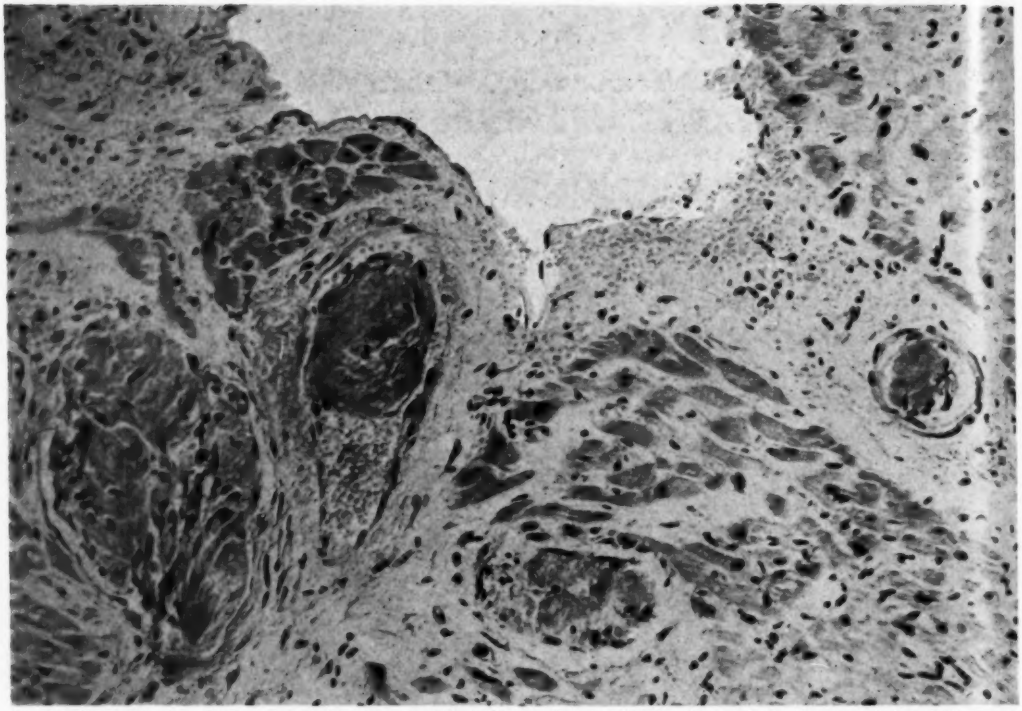

FIG. 2.—High-power view of typical thrombotic microangiopathy in four small myocardial blood vessels in another case of thrombotic purpura. (Haematoxylin-eosin.)

logical abnormalities were felt to be inconsistent with the imminent development of such a striking and rapidly mortal illness as thrombotic purpura: the histological findings were therefore discounted. It was not until three weeks later that the unmistakable clinical syndrome of the disease developed. The diagnosis was confirmed histologically after death. Publication of this case has unfortunately been the cause of some diagnostic confusion, for it has led to banal thrombosis in curettings being misinterpreted as evidence of incipient thrombotic purpura. It seems therefore desirable to write this note in order to draw attention to the possible difficulties, which in fact should be quite readily avoidable.

The paper in which the case report appeared was intended to be essentially clinical in its approach to the role of biopsy in the diagnosis of thrombotic purpura. Histological differential diagnosis was not discussed, having been mentioned, although only briefly, in earlier papers (Symmers, 1952, 1953). The potential frequency of misinterpretation of endometrial vaso-thrombosis began to become familiar through the worrying experiences of colleagues for whom banal thrombosis in curettings had taken on a troublesomely equivocal significance. These experiences made it seem advisable, when working on a general review of the histological diagnosis of some of the so-called "collagen diseases," to say, "Even typical histological lesions do not alone justify the diagnosis [of thrombotic purpura], for they may be closely simulated in some other conditions: thus, these lesions may be mimicked by thrombosed endometrial capillaries and venules in association with haemorrhagic endometrial hyperplasia. . . ." (Symmers, 1958). Banal thrombosis in dilated endometrial blood vessels can in fact simulate the appearances of the typical thrombotic microangiopathy of thrombotic purpura closely enough for mistakes to occur quite readily. The vessels in the superficial parts of the hyperplastic endometrium, particularly venules, are those usually affected; the endometrial hyperplasia may be due to metropathia haemorrhagica or associated with uterine

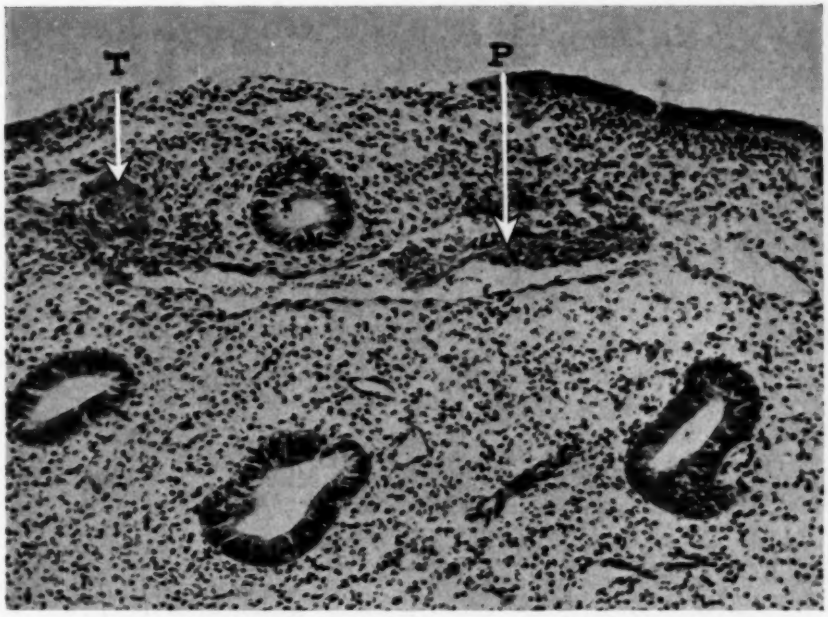

FIG. 3.—The thrombus (T) in the dilated superficial blood vessel in this piece of curetted endometrium from a patient under treatment with an oestrogen was shown in serial sections to be continuous with the seemingly free-lying thrombus (P) within the lumen. The lumen was nowhere completely occluded. The propagated part of the thrombus had acquired a partial covering of endothelium. Dilatation and incomplete occlusion of affected vessels, propagation of thrombus within their lumen, and endothelialization of the surface of the thrombus are usually characteristic of the lesions of thrombotic purpura, and the thrombi do not usually contain much, if any, fibrin. The thrombus illustrated here consisted mainly of fibrin. However, the main factor enabling thrombotic purpura to be excluded in this case was the subsequent failure of any other evidence of this disease to develop. (Haematoxylin-eosin.)

fibroids, oestrogen-secreting ovarian tumours, or treatment with oestrogens. In my own experience it has been in the last-mentioned category that this type of thrombosis has been met with most often, and particularly in cases of metrorrhagia in women having treatment with oestrogens for menopausal or postmenopausal disturbances. To some extent, of course, any piece of tissue, small biopsy specimens in particular, in which there is thrombosis of small-calibre blood vessels is liable to comparable misinterpretation.

Paradoxically, mistakes in interpretation of the significance of these banal thrombotic lesions seem likelier to be made by those who have seen the characteristic and widely disseminated vascular lesions of thrombotic purpura in necropsy material. It is as if the observer who is familiar with the striking overall histological picture of thrombotic purpura (Fig. 1) and with the appearance under higher magnification of its individual lesions (Fig. 2) comes to be particularly on the lookout for the presence of any thrombotic lesions in small vessels, and then falls too easily into the trap of dogmatically interpreting all sorts of thrombotic lesions as lesions of thrombotic purpura (Figs. 3 and 4). It is clearly imperative that the histological appearances should always be interpreted in the light of the clinical and haematological findings. A diagnosis of incipient thrombotic purpura on the basis of biopsy findings alone, in other words, as a result of unexpectedly finding an apparently typical thrombotic microangiopathy when there is no clinical and haematological evidence of the disease, demands the greatest circumspection: a vascular lesion which would be acceptable in support of a well-founded clinical diagnosis of thrombotic purpura would have far less diagnostic value when there were no confirmatory clinical and haematological manifestations. Thrombotic purpura can be diagnosed from biopsy material alone only when multiple, typical lesions are present, and never safely on the basis of an isolated finding in a single vessel, although any

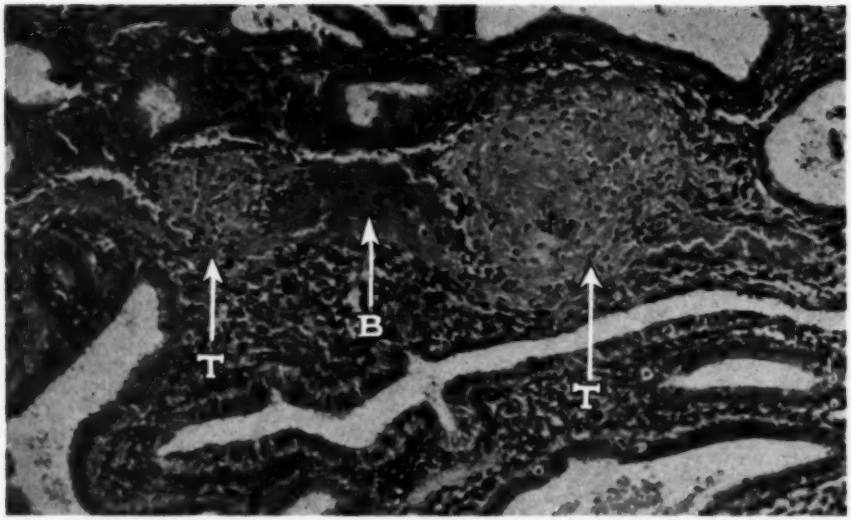

FIG. 4.—The thrombi (T, T) in the dilated vessel in this specimen of endometrium look deceptively like banal thrombi. The patient was already developing the full syndrome of thrombotic purpura when the curettage was performed. Microaneurysm formation without necrosis of the vessel wall or angeitis is a characteristic feature of the thrombotic microangiopathy of thrombotic purpura. There is some degree of microaneurysmal accentuation of the dilatation of the vessel shown here, particularly at the sites of the thrombi, but in fact such dilatation has less diagnostic significance when the vessels affected are such thin-walled and naturally distensible ones as those of endometrium. (B shows blood cells in the engorged and only incompletely obstructed lumen.) (Haematoxylin-eosin.)

such findings must always alert the observer to the possibility of this disease, which has to be recognized early in its course if there is to be the least prospect of treating it successfully.

These remarks could be expanded by discussing the differential diagnosis of thrombotic microangiopathy in detail, but as most of the simulant conditions have been listed in the papers mentioned above it is not proposed to refer to them again here. It is, however, worth stressing that leucocytic infiltration, vasonecrosis, and fibrin-rich thrombi are so very exceptional in the histological picture of thrombotic purpura that their presence in biopsy material should be considered as evidence against this diagnosis.

From the practical point of view, endometrium is the tissue most frequently seen in the biopsy laboratory in which potentially confusing vascular changes are least uncommon, and for this reason it has been chosen as the subject of this note. The principles illustrated by this account are applicable, *mutatis mutandis*, to other tissues in which thrombosis of the small blood vessels presents a problem in diagnostic interpretation.

Summary

Attention is drawn to the possibility of mistaking banal thrombosis in small blood vessels in biopsy specimens for the thrombotic microangiopathy of thrombotic purpura. This misinterpretation has been made most frequently in cases of simple endometrial hyperplasia, and may have been encouraged by the author's account (Symmers, 1956) of a case of thrombotic purpura in which the diagnosis was first disclosed by the finding of the typical vascular lesions in a specimen of uterine curettings. Biopsy can prove invaluable as a means of confirming a clinical diagnosis of thrombotic purpura. It is only exceptionally, however, that biopsy findings on their own can warrant making this diagnosis in the absence of supporting clinical and haematological evidence.

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J. clin. Path. (1959), **12**, 561.

CYTOLOGICAL PREPARATIONS FROM PROTEIN-FREE FLUIDS

BY

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It is necessary for the preparation of stained smears for cytological examination that cells be made to adhere to glass slides. From sites such as the vagina, cervix, oral cavity, and bronchial tree this is a simple matter, as the fluid portion of the specimen contains sufficient protein material to induce good adhesion to the slide when treated with a suitable fixative. If the fluid portion of the specimen is protein-free it is much more difficult to persuade the cells to stick to the slide, many of them floating off when the slide is placed in the fixative. For this reason the cytological examination of urine, and to a lesser extent of pleural fluid, has always presented a problem, and various methods have been employed in order to obtain satisfactory slides showing reasonable numbers of cells. The majority of workers use Papanicolaou's method (Papanicolaou, 1954), in which a centrifuged sediment of urine is mixed with albumin, smeared on to glass slides previously coated with an albumin film, and fixed in an alcohol-ether solution. The smears are said to adhere better if they are allowed to dry at the edges before fixation and a coating of celloidin is applied before staining. Other workers have used substances other than albumin for adding to the sediment to promote adhesion, including pectin (Rofe, 1955), leuconostoc cultures and human saliva (Sagi and Mackenzie, 1958), and glycerine-albumin (Booth, 1959). Another method is the use of frosted glass slides (Dakin, 1955).

The purpose of this paper is to describe an entirely different method which is simple to use and gives preparations with excellent distribution of cells in adequate numbers. The basis of the method is the direct centrifugation of the cells on to a coverslip previously coated with an adhesive which retains its stickiness under water. The coverslip is held in a " perspex " cell containing the cell suspension and centrifuged with the sticky surface of the coverslip at right angles to the radius of the centrifuge. The apparatus used was originally designed as part of a technique for the differential separation of malignant cells from blood, but for various reasons has proved of little value for this purpose.

A special centrifuge head is an essential feature of the technique, and this will be described before recording the details of the method itself.

Centrifuge Head

This was designed to fit on to an M.S.E. " minor " centrifuge. Fig. 1 shows a part-sectioned elevation and plan views of the head, and Fig. 2 the head with

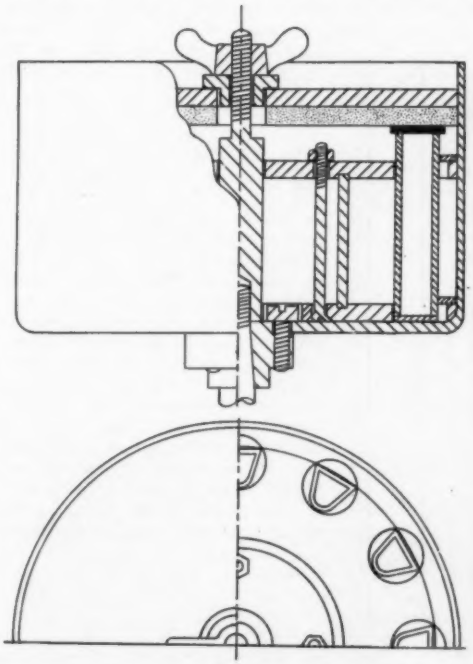

FIG. 1

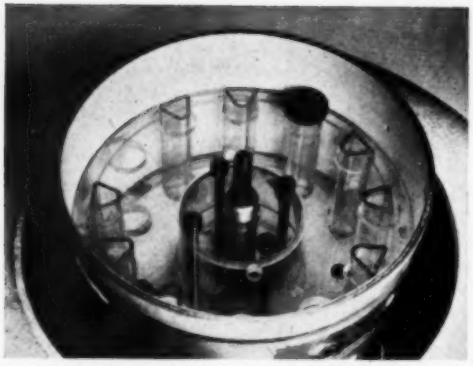

FIG. 2.—Centrifuge head with lid removed. Only one rubber washer is shown.

the lid removed. The bowl was made from an aluminium saucepan (of the deep-drawn, not cast, variety) to which a brass boss was fitted having a tapered bore to suit the centrifuge spindle. A "perspex" framework was fitted into the bowl to hold 12 specially designed centrifuge tubes. This consists of two ¼-in. thick discs separated by a length of 2½-in. diameter "perspex" tubing, fixed together to form a single unit by four bolts. Each of the discs has 12 ¾-in. diameter holes equally spaced around the periphery, leaving a margin of 3/16 in. between their outer edges and the edges of the discs. A ring of 1/16 in. thick "perspex," 5/16 in. wide, is cemented to the outer part of the discs so that it partly intersects the holes, converting each of them into a D-shape. The centrifuge tubes are triangular in section and also made of "perspex." Details of one of these are shown in Fig. 3. The dimensions of the tubes provide that the flat vertical surface opposite the rounded corner takes a 2½ in. × ½ in. coverslip as a loose sliding fit when inserted as shown in the drawing. These tubes fit loosely into the holes in the "perspex" framework as shown in Figs. 1 and 2. The centrifugal force keeps them in contact with the narrow peripheral ring, which ensures that the surfaces of the coverslips are accurately held at right angles to the radius of the head. The open ends of the tubes are sealed during centrifuging by individual circular rubber washers held in position by a separate ¼-in. thick "perspex" disc clamped down by a single wing nut on to the threaded rod fitted to the central spindle. A foam rubber pad glued to the under side of this disc gives even pressure on all the tubes.

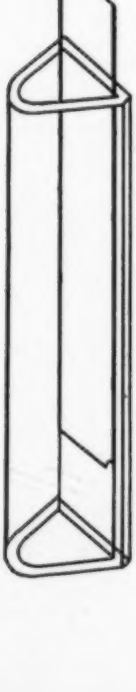

FIG. 3.—"Perspex" cell with coverslip partly inserted.

Method

Preparation of Coverslips.—Rectangular No. 2 coverslips measuring 2½ in. × ½ in. are used. A small portion of one corner of each coverslip is snapped off (see Fig. 3) in order to facilitate removal from the "perspex" cell. The coverslips are thoroughly washed with soap and warm water, rinsed in water, and dried on a clean cotton cloth.

The adhesive used is "evostick" impact adhesive (PSM 317), which is obtained as a thick syrupy fluid and must be diluted 1:5 in benzene. The coverslips are coated on one surface with this solution, applying it fairly liberally with a camel-hair paintbrush and allowing them to dry in a dust-proof container. They will remain tacky indefinitely. If the adhesive is applied so that the snapped-off corner is always in the same position, this notch also serves to identify the adhesive side of the coverslip during fixing, staining, and mounting.

The prepared coverslips are placed in the "perspex" centrifuge tubes with the broken corners to the bottom of the tubes and the adhesive surfaces facing the rounded sides of the tubes. The triangular section of the tube prevents the coverslip from falling away from the outer wall before centrifuging. The tubes are then ready to receive the cell suspension.

Preparation of Cell Suspension.—Most specimens of urine contain a mucoid substance which will prevent the cells from sticking to the adhesive coverslips, and this is first of all reduced to a negligible amount by a simple washing process.

The urine sample is spun in any suitable centrifuge tubes at 1,500 r.p.m. for five minutes. The supernatant fluid is sucked off and replaced by an equal volume of normal saline. The sediment is well mixed with the saline by inversion and then spun again as before. The supernatant is again removed and a further volume of saline added to the sediment and the two well mixed. The volume of the last addition of saline may vary within wide limits; it should theoretically be such that after preparation of the slide the distribution and number of the cells are ideal for ease of scanning and cell recognition, and obviously depend on the concentration of cells in the original sample. In practice, however, it has been found that satisfactory preparations are obtained from most urine samples if the volume of the final saline addition is equal to about one-tenth of the original volume of urine. The "perspex" tubes hold about 6 ml. of fluid, and this amount must therefore be a minimum. If the specimen is from a woman for examination of anterior urethral cells an original:final volume ratio of between ½ and 1

has been found satisfactory. After mixing the sediment with the final volume of saline, the suspension is transferred to the triangular plastic tubes which already have the adhesive coverslips in position and placed in the special centrifuge head. The individual rubber washers are placed over the open ends of the tube and the lid screwed firmly into position. The tubes are spun at 3,000 r.p.m. for 10 minutes. The coverslips are then removed from the tubes by means of a straight stiff wire with a small right-angled hook at one end which is passed to the bottom of the tube and hooked on to the broken corner of the coverslip. They are then immediately placed in the fixative.

Fixation, Staining, and Mounting.—Because the cells are adhering to an adhesive which is easily removed by organic solvents, fixatives and mounting media containing ether or xylol or having a high alcohol content must be avoided, and certain modifications of the common methods are used. Any watery fixative, such as formal saline or formal sublimate, can be used, but the best results were obtained using a modified Schaudinn's fixative with a lowered alcohol content, prepared by mixing equal quantities of absolute ethyl alcohol and a saturated aqueous solution of mercuric chloride. The adhesive is not attacked by this fixative provided the coverslips are left in it for two minutes only. The cells are well fixed in this time.

For routine work we use haemalum and eosin for staining. Papanicolaou's stains cannot be used owing to their high alcohol content. For studies of the female anterior urethra Shorr's stain is satisfactory. The counterstaining portion of Shorr's stain contains 50% alcohol, and the coverslips must not be left immersed for more than two minutes.

The common xylol- or turpentine-containing mounting media cannot be used alone because they dissolve the adhesive and produce clumping and distortion of the cells. Other media, such as polystyrene in mesitylene, do not appear to affect the adhesive in the same way as xylol but produce a haziness in the preparation together with some cell shrinkage. After trying various media it was found that a watery medium followed by colophonium in turpentine gave excellent results.

Following the final wash after staining, the coverslip is immediately flooded with a 15% solution of polyvinyl pyrrolidone (P.V.P.), drained, and allowed to dry with the adhesive surface uppermost. When dry the coverslip is mounted on a 3×1 in. slide with xylol-colophonium in the usual way. The acid dyes tend to be removed from the cells when the P.V.P. solution is added, but this does not occur if the solution is made slightly acid by the addition of acetic acid to *p*H 5–6.

Results

The method has given excellent results in examination of urine in cases of carcinoma of the bladder. Whether cytological examination of urine for cancer cells is a worth-while process in view of the ease of cystoscopy and biopsy in hospital practice is a matter for debate. The method may perhaps be more useful in routine examination of urines from aniline dye workers. A useful application is for the examination of female urine for studies of hormone effects on the squame cells of the anterior urethra. These cells show parallel changes to those of the vagina (*Brit. med. J.*, 1959) and provide an easily obtained source of material which can be taken by the subject herself as often as necessary. For this purpose slides are required containing large numbers of cells with a good distribution, and this has formerly been difficult to obtain for the reasons already mentioned.

It is often necessary to use urine as a sample in the preparation of prostatic cell smears, using the first few millimetres passed after massage. In this case the natural adhesive property of the prostatic secretion is greatly diminished and the adhesive slide method may be used to advantage.

The method also gives good preparations from pleural fluids. Some specimens have quite a high

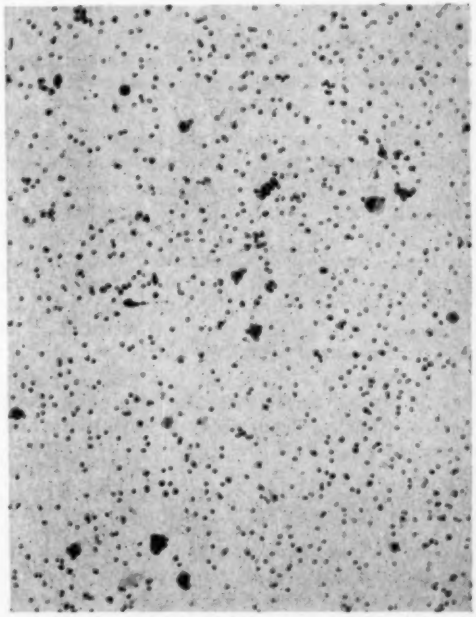

FIG. 4.—Urine preparation from case of carcinoma of bladder. × 150.

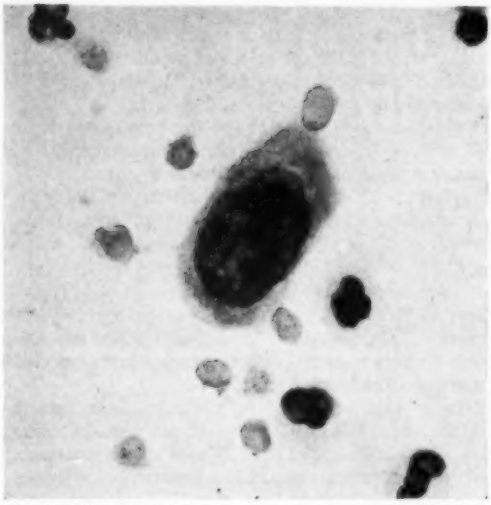

FIG. 5.—Malignant cell in urine from case of carcinoma of bladder. × 2,000.

protein content and may clot, in which case it is best to remove the clot after it has contracted and to treat the remaining fluid as described for urine.

Figs. 4–6 show examples of the preparations obtained.

Fig. 4 is a low-power view of a preparation made from urine of a patient with a known carcinoma of the bladder and stained with haemalum and eosin. The field included in the picture represents $\frac{1}{2,500}$ of the area of the coverslip, and it is stressed that this distribution and cell density are of the same order over the whole area of the slide. In addition, the results are consistent, whereas the usual albumin technique gives inconsistent results with varying numbers of cells on different slides prepared from the same specimen, with a very irregular distribution of cells which makes scanning awkward.

Fig. 5 shows a malignant cell from a known case of bladder cancer and is included to show the degree of nuclear detail obtained with the method.

Fig. 6 shows a high-power view of anterior urethral cells prepared from normal female urine on the 25th day of a 32-day menstrual cycle and stained with Shorr's stain. The degree of cornification, nuclear variation, and curling of the cell margins are apprehended with the same ease (or difficulty) as with smears made in the usual way, but counting the number of cells is simplified by their regular distribution.

FIG. 6.—Urine preparation from normal female. × 600.

Summary

A method of preparing specimens for cytology from protein-free fluids is described wherein the cells are directly centrifuged on to adhesive-coated slides. The method has great advantages over the usual process in that the number of cells retained on the slide is greatly increased together with a very even distribution.

I am grateful to Professor H. A. Magnus for his encouragement and helpful criticism in the preparation of this paper; also to Mr. G. A. Harwood for much technical advice, labour, and enthusiasm, and to Miss M. T. Pickett, research assistant in the Department of Endocrinology, Chelsea Hospital for Women, for supplying preparations of anterior urethral cells.

Messrs. Evode Ltd., of Stafford, kindly supplied the adhesive material used in this method.

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THE BACTERIAL FLORA OF GASTRIC LAVAGES FROM PATIENTS UNDERGOING PARTIAL GASTRECTOMY

BY

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Several investigations have been made into the bacterial content of the stomach and small intestine in health and disease.

Cregan and Hayward (1953) investigated the bacterial content of the healthy human small intestine by aspiration through the gut wall, and, on the few occasions when growth was present, found only transient flora, mainly mouth species. Anderson and Langford (1958) confirmed these findings, and found no resident flora in cases of coeliac disease and fibrocystic disease of the pancreas.

Howie, Duncan, and Mackie (1953) cultured stomach washings for *Cl. welchii* after partial gastrectomy in view of the similarity between the clinical symptoms of post-gastrectomy diarrhoea and of the gastro-enteritis caused by food-poisoning with heat-resistant *Cl. welchii*, type A (Hobbs, Smith, Oakley, Warrack, and Cruickshank, 1953; Oakley and Warrack, 1953). They found numerous *Cl. welchii* in stomach washings from 12 of 15 patients examined within a week after partial gastrectomy. In eight of the 15 patients, small amounts of *Cl. welchii* α toxin were also demonstrated in the same specimens.

Duncan, Goudie, Mackie, and Howie (1954) took specimens of jejunal contents by Miller-Abbott tube from eight patients with anaemia or failure to gain weight after partial gastrectomy. They could find no evidence of bacterial colonization in the jejunum of such patients. They also found heavy growths of *Cl. welchii*, type A, in the stools of five patients with post-gastrectomy diarrhoea and in a case of acute obstruction of the small intestine. Diarrhoea caused by *Cl. welchii* as a result of food poisoning (McNicol and McKillop, 1958) does not come within the scope of this paper.

The present experiment was based on a series of 60 partial gastrectomies performed by Mr. T. G. E. Loosemore. Specimens were collected from the indwelling Ryle's tube pre-operatively and for a period of 24 to 30 hours after operation. Of the 60 operations, 51 were performed on waiting-list cases for chronic gastric ulcer and duodenal ulcer, six were emergency operations for perforated ulcers, two were emergency operations for haematemesis and melaena, and one radical partial gastrectomy was performed on a case of suspected gastric ulcer, where a small prepyloric carcinoma was found. All patients were given a prophylactic course of "distavone"* (combined penicillin and dihydrostreptomycin) post-operatively.

Method

Gastric lavage specimens were taken from the 60 cases reviewed at the following times: (*a*) Pre-operatively, except in certain of the emergency cases; (*b*) hourly for the first six hours after operation; (*c*) four-hourly thereafter for a period varying from 18 to 24 hours.

The specimens were cultured on horse blood agar plates, incubated aerobically and anaerobically, MacConkey plates, and 7% salt plates. The specimens were also inoculated into cooked meat media, and subcultured on to Nagler plates after 48 hours' incubation. To facilitate isolation of *Cl. welchii*, the Nagler plates contained neomycin (Lowbury and Lilly, 1955).

All organisms growing on the plates were identified. The antibiotic sensitivities of all staphylococci isolated were determined, and any penicillin-resistant staphylococci were phage typed. From Case 43 onwards, throat swabs were taken routinely on the third or fourth day after operation.

Results

Twenty-three of the pre-operative aspirations were sterile. All these were from cases of chronic gastric ulcer or duodenal ulcer. In 19 of these cases subsequent aspirations grew *Bact. coli*. This organism appeared three to four hours after operation. Post-operative aspirations grew mixed throat flora in three of the cases with a sterile pre-operative specimen, and, in the remaining

*Distavone (Distillers Co. Ltd.) is a mixture of procaine penicillin G which is sparingly soluble in water, and potassium penicillin G which dissolves readily. On administration there is an initial "loading" dose plus a prolonged "depot" dose of penicillin. A third constituent, dihydrostreptomycin, is effective against Gram-negative organisms and acts synergistically with the penicillin against the Gram-positive cocci.

case, the aspirations remained sterile post-operatively.

Table I shows the preponderant bacterial growths isolated from the gastric washings during specified periods of time. The figures are

TABLE I
PREPONDERANT GROWTH OF BACTERIA AT SPECIFIED TIMES CALCULATED IN 60 CASES

Organism	Pre-operative (%)	1–4 Hours (%)	5–10 Hours (%)	11–16 Hours (%)	17–24 Hours (%)	Over 24 Hours (%)
Bact. coli ..	25	63	80	70	65	21
Staph. aureus ..	5	18	13	18	16·5	8·5
Strep. viridans ..	5	11	10	7	3·5	3·5
,, *haemolyticus*	3·5	7	5	7	3·5	1·5
Strep. faecalis ..	5	11	10	7	7	5
Paracolon bacilli	1·5	5	3·5	1·5	0	0
Other staphylococci ..	0	0	1·5	0	0	0
Cl. welchii ..	0	1·5	0	3·5	7	0
H. parainfluenzae	1·5	7	5	1·5	1·5	0
Ps. pyocyanea ..	0	1·5	1·5	1·5	1·5	1·5
Micrococci ..	5	17	10	10	11·5	1·5
Proteus	3·5	10	15	16·5	18	8
Neisseriae ..	0	3·5	1·5	1·5	3·5	1·5
Mixed throat flora	3·5	15	18	15	13·5	0
Yeasts	0	3·5	1·5	3·5	1·5	5

expressed as the percentage of the total number of cases which grew a certain organism at a certain time. It will be seen that *Bact. coli* was by far the most common organism found, with *Staph. aureus* coming second. There was a relatively high incidence of upper respiratory tract flora, which were presumed to come from swallowed saliva.

Cl. welchii was found in the washings of only five cases. Four cases in the series had post-gastrectomy diarrhoea, but in none of the washings from these cases was *Cl. welchii* isolated. In two of these cases, the diarrhoea was thought to be a result of vagal crush, and in one it was staphylococcal in origin. No cause could be found for the diarrhoea in the other case.

Two cases developed infected wound sites. From one, *Staph. aureus* was isolated, and, from the other, a mixed growth of paracolon bacilli and coccobacilli.

Table II gives the results of cultures in the 18 cases in which throat swabs were taken. It will

TABLE II
RESULTS OF THROAT SWABS IN 18 CASES

Organisms	No. of Cases	Percentage of Total
Mixed throat flora	9	50
Staph. aureus	6	33
,, ,, phage type 80 ..	5	28
Strep. viridans	4	22
H. parainfluenzae	3	17
Proteus	1	6
Strep. haemolyticus	1	6

TABLE III
RESULTS RELATING TO *STAPH. AUREUS*

Cases in which *Staph. aureus* was isolated from throat swabs	6
Cases in which *Staph. aureus* was isolated from gastric aspirations	26
Cases in which *Staph. aureus* was isolated from washings alone	21
Cases in which *Staph. aureus* was isolated from throat swabs alone	1
Cases in which *Staph. aureus* was present both in washings and throat swabs	5

be seen that there is a relatively high incidence of *Staph. aureus*, and, in five cases out of six, the organism was of phage type 80.

Table III compares the incidence of *Staph. aureus* in throat swabs and gastric aspirations. In all the cases except one, in which *Staph. aureus* was isolated from the throat, it was also isolated from the stomach washings. In two cases, the organisms in the stomach and the throat were of the same phage type. One of these cases, in which both the throat swab and gastric aspirations contained *Staph. aureus*, phage type 80, was of interest because no evidence of gastric ulcer was found at operation but microscopical sections showed many pus cells in the gastric mucosa.

In the third case, the organisms from both sites were different strains. In the fourth case, *Staph. aureus*, phage type 80, was cultured from the throat swab. The gastric washings from this case contained a penicillin-sensitive *Staph. aureus* up to 12 hours, but thereafter a penicillin-resistant *Staph. aureus*, phage type 80, replaced it.

The fifth case is of special interest, and deserves a fuller description:

J. D., a man aged 50, was admitted with a history of indigestion for many years, and distension and vomiting for eight weeks. At operation on January 30, 1958, a small carcinoma was found in the pre-pyloric region, and a radical partial gastrectomy performed. Post-operative progress was good. A *Staph. aureus* was found in the post-operative gastric aspirations which was not phage type 80.

On January 21, 1958, another patient, G. E., had been transferred to the same ward for an emergency ileostomy to relieve severe ulcerative colitis. He occupied a bed on the other side of the ward, almost directly opposite to the patient J. D. On January 27 culture of the stool of G. E. yielded a *Staph. aureus*, phage type 80, which was resistant to erythromycin.

A routine throat swab from J. D. on February 4 yielded a *Staph. aureus*, phage type 80, which was also resistant to erythromycin. On February 11, J. D. developed a transitory attack of diarrhoea (the only complication in an otherwise uneventful post-operative course), and culture of his stool yielded a heavy growth of *Staph. aureus*, phage type 80, which was of similar sensitivity pattern to the one in his throat.

Patients were also grouped as to whether they had post-operative fevers or not. A patient assumed to have had a post-operative fever was taken as one developing a temperature of at least 100° F. on at least two occasions. Table IV gives the results of this investigation. It will be seen that the incidence of "distavone"-resistant *Staph. aureus* in cases with post-operative fever was over four times that in cases without fever.

TABLE IV
POST-OPERATIVE FEVERS

Total cases in series	60
Cases in which *Staph. aureus* was isolated	26
Percentage of cases in which *Staph. aureus* was isolated	43
" " " " " "distavone"-resistant *Staph. aureus* was isolated	20
Cases with post-operative fever	16
Percentage of fevers in total number of cases	26·7
Post-operative fevers in which *Staph. aureus* was isolated	14
Percentage of post-operative fevers in which *Staph. aureus* was isolated	87
Percentage of post-operative fevers in which "distavone"-resistant *Staph. aureus* was isolated	50
Cases without post-operative fever	44
" " " " in which *Staph. aureus* was isolated	12
Percentage of cases without post-operative fever in which *Staph. aureus* was isolated	28
Percentage of cases without post-operative fever in which "distavone"-resistant *Staph. aureus* was isolated	11

Discussion

It must be assumed that organisms such as streptococci and neisseriae are present in gastric aspiration specimens as a result of the patient swallowing saliva containing these organisms.

In an attempt to determine the source of other organisms found in the aspiration specimens, phenolphthalein phosphate plates containing penicillin and Nagler plates were exposed in a surgical ward. Very few colonies of *Bact. coli* and *Proteus* were found after incubation, and one can only assume that these organisms were present in the aspirations as a result of reflux from the gut.

Large numbers of *Staph. aureus* were found on the exposed plates, and these obviously make their way to the stomach by inhalation and by swallowing of saliva and upper respiratory tract secretions.

No relationship was found between the presence of *Cl. welchii* in the aspirations and post-operative diarrhoea. If the *Cl. welchii* isolated came by reflux from the gut, they were probably not toxogenic strains. It is quite possible that they were inhaled and swallowed organisms, in view of the fact that moderate numbers of colonies were obtained from the exposed Nagler plates.

From Table IV it may be assumed that the prophylactic use of "distavone" post-operatively is useful therapeutically, since *Staph. aureus* was isolated from 43% of cases, whereas only 26.7% of cases developed post-operative fevers. The high incidence of "distavone"-resistant *Staph. aureus* in the cases of post-operative fever suggests that many of these fevers were staphylococcal in origin.

Conclusion

The post-operative procedure of aspirating gastric contents for removing blood is fraught with the possible hazard of introducing throat and aerial flora via the Ryle's tube into a bacterial-nutrient medium, but the advantages gained by aspiration outweigh the risk involved. Aspiration removes stale, fermenting blood and so prevents this foetid material passing into the intestines where antibacterial acid is absent. This may account for the incidence of only one case (J. D.) of post-operative staphylococcal enteritis out of 26 patients from whom *Staph. aureus* was isolated in post-operative gastric secretions.

The patient, J. D., had been exposed to a heavy dissemination of staphylococci from another patient, G. E. Barrier nursing of septic cases, dressings changed in a special nursing room designed to avoid both contact and air-borne infection (Colebrook, 1958), strict adherence to the no-touch technique of dressing, and freshly laundered cotton blankets (Schwabacher, Salsbury, and Fincham, 1958), are some of the essential precautions to prevent staphylococcal enteritis.

Summary

The bacteriological findings in 60 cases undergoing partial gastrectomy are reviewed.

Twenty-six of the cases were infected with *Staph. aureus*.

Only one case had staphylococcal diarrhoea. There was no evidence to relate the presence of *Cl. welchii* in the washings and post-operative diarrhoea.

The value of post-operative gastric aspiration is discussed.

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NOVOBIOCIN-TETRATHIONATE BROTH: A MEDIUM OF IMPROVED SELECTIVITY FOR THE ISOLATION OF SALMONELLAE FROM FAECES

BY

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The selective cultivation of bacteria by incorporating chemicals in the media depends on supplying a substance which either stimulates the growth of the species to be isolated or inhibits unwanted species. Tetrathionate is a good selector of Salmonellae in faeces culture, and is believed to act in both ways. Tetrathionate reducers, such as Salmonellae, flourish in broth containing it, whereas a high concentration of tetrathionate inhibits many other faecal organisms (Knox, Gell, and Pollock, 1943). Unfortunately, however, members of the Proteus group are powerful tetrathionate reducers, and when they are present selection of Salmonellae is much impaired, consequently the addition of a further selective inhibitor effective against Proteus organisms is desirable. So far no such satisfactory substance has been found: brilliant green and ox bile (Kauffmann) proved unsuccessful (Knox *et al.*, 1942, 1943).

Antibiotics are becoming increasingly used as selective agents in culture media, but most of them are approximately equally active or inactive against Salmonellae and Proteus. Novobiocin, however, inhibits Proteus more than other Gram-negative bacilli and seemed therefore a suitable substance for trial.

Materials

Tetrathionate Broth.—This was prepared by adding two "oxoid" tablets to 10 ml. of water in a 1 oz. screw-capped bottle and bringing to the boil. The medium without iodine can be stored at room temperature for one month; once iodine is added the complete medium is unstable.

Novobiocin-tetrathionate Broth.—Novobiocin solution containing the appropriate concentration in 0.1 ml. was added to 10 ml. of the incomplete medium, 0.2 ml. of the specified iodine solution being added before use.

Novobiocin Solution.—The sodium salt of novobiocin (Upjohn of England Ltd.) was used, and the strengths of all solutions were expressed as concentrations of pure novobiocin. As the novobiocin content varied with different batches of the salt a simple calculation was necessary each time a new sample was used. A stock solution containing novobiocin, 16,000 μg./ml. of water, was stored in the refrigerator.

MacConkey Agar.—Oxoid.

Desoxycholate-citrate Agar. — Southern Group Laboratory (Lewisham).

Indicator Serum Broth.—This was prepared by the method of Stokes (1955).

Investigation

For efficient selection a concentration of novobiocin is required which will inhibit most, if not all, strains of Proteus without jeopardizing the survival of small numbers of Salmonellae.

Sensitivity to Novobiocin.—The sensitivities to novobiocin of 27 strains of Proteus and of 24 strains of Salmonellae were tested by the paper disc method and by tube titration. Proteus was always more sensitive than Salmonella (Table I).

TABLE I

NOVOBIOCIN SENSITIVITY TESTS

Organism	No. of Strains Tested	Disc Test		Tube Titration (Inhibitory Concentration of Novobiocin in μg./ml. Indicator Serum Broth)	
		Sensitive	Resistant	Average	Range
Salmonella species	24	—	24	>2,400	1,600–3,200
P. morganii ..	5	5	—	85	25–200
P. mirabilis ..	13	13	—	170	25–400
P. vulgaris	9	9	—	160	25–400
Paracolon bacilli ..	5	—	5		
E. coli	5	—	5		
Ps. pyocyanea ..	4	—	4		

Effects of Concentrations of Novobiocin in Tetrathionate Broth upon Growth of Salmonellae.—A plate viable count (Miles and Misra, 1938) was made on blood agar from an overnight broth culture of *Salmonella paratyphi B.* Drops from the highest dilutions were seeded into bottles containing

TABLE II

RECOVERY OF SALMONELLAE FROM ARTIFICIALLY INFECTED FAECES SUSPENSIONS CONTAINING PROTEUS

Experiment	Salmonella	Proteus		Minimal Nos. of Salmonellae/ml. of Faeces Suspension Detectable after Culture in			
		Strain	Approximate No./ml. Faeces	Tetrathionate Broth	Proteus Present	Novobiocin-tetrathionate Broth	Proteus Present
a	*Schwarzengrund*	*Morganii* (11,229)	5,000	42×10^6	+++++	7	+
b	,,	,,	266,000	$>28 \times 10^3$	+++	28 or less	
c	,,	*Vulgaris*	5,000	42 or less	+++	42 or less	
d	*Typhi-murium*	*Mirabilis* (7,406)	5,000	$>140 \times 10^6$	+++++	140 or less	++++
e	*Paratyphi B*	,,	5,000	88×10^6	+++++	88×10^4	++
f	*Enteritidis*	*Morganii*	5,000	22×10^4	+++++	22×10^4	
g	*Thompson*	,,	5,000	30 or less	+++++	30 or less	
h	*Anatum*	*Mirabilis*	5,000	34×10^3	+++++	35 or less	

+ Indicates the number of bottles in which Proteus survived.

tetrathionate broth and into bottles containing different dilutions of novobiocin in tetrathionate broth. Each concentration of the organism was tested in triplicate in each medium. The test was repeated with the same strain of *Salmonella paratyphi B* and also with a strain of *Salmonella enteritidis*.

As a result of these tests a concentration of 40 μg./ml. novobiocin in tetrathionate broth appeared to be the most suitable, since there was no inhibition when growth of about 3 to 30 organisms was compared in tetrathionate broth and tetrathionate broth plus 40 μg./ml. novobiocin.

Suppression of Proteus by Tetrathionate Containing Novobiocin.—Diluted overnight broth cultures of four strains of Proteus were seeded into triplicate bottles of nutrient broth, tetrathionate broth, and novobiocin tetrathionate broth. Novobiocin at a concentration of 40 μg./ml. completely suppressed growth of an inoculum of about 70 organisms during incubation overnight.

Stability of Novobiocin in Selective Media.—It seemed likely that if novobiocin proved to be a useful selector in tetrathionate broth it might also prove of value in other enrichment media for Salmonellae. Its stability was therefore tested in tetrathionate broth, Selenite-F medium, and in the medium of Rappaport, Konforti, and Navon (1956).

The drug was stable at a concentration of 40 μg./ml. in all three media for at least 48 hours at 37° C. and for at least one month at 4° C. There was no loss of potency in tetrathionate broth on storage without iodine at room temperature for one month. Novobiocin at a concentration of 40 μg./ml. tetrathionate broth was used in all subsequent experiments.

Comparison of Tetrathionate and Novobiocin-tetrathionate Broths for Recovery of Salmonellae in Presence of Proteus from Artificially Infected Faecal Suspensions.—An emulsion was prepared in 40 ml. of water of approximately 5 g. of faeces from a patient who was not receiving antibiotics. A dilution of an overnight broth culture of Proteus was added to give approximately 5,000 bacilli/ml., and 5 ml. of this suspension was delivered into each of 5×1 oz. screw-capped bottles. Counted numbers of Salmonellae were then added to give a series of bottles containing faeces, a constant number of Proteus, and varying numbers of Salmonellae. Each bottle was shaken thoroughly on a mechanical shaker, and 1 ml. quantities from each bottle were cultured in tetrathionate broth with and without novobiocin.

After overnight incubation subcultures were made on MacConkey agar and two colonies of each type of non-lactose-fermenter were tested for urease production. Those which proved to be urease negative were tested by slide agglutination, using the appropriate Salmonella antiserum. As the properties and colonial appearances of the Salmonella strains were well known, final identification by tube agglutination was considered to be superfluous. The number of each type of non-lactose-fermenting colony examined was purposely limited so that the chance of isolating Salmonellae should be approximately the same as it would be in routine examinations of faeces.

Table II shows that Salmonellae representative of groups B, C, D, and E can be isolated more successfully by enrichment in novobiocin-tetrathionate broth than in plain tetrathionate broth when Proteus is present.

Group A was not investigated because the strain of *Salmonella paratyphi A* examined proved to be inhibited by tetrathionate broth, which confirmed the findings of Banwart and Ayres (1953).

Comparison of the results of experiments (a) and (b) shows that the efficiency of novobiocin-tetrathionate broth may be unaffected by a 50-fold increase in the number of Proteus.

In experiments (c), (f), and (g), novobiocin-tetrathionate broth did not detect smaller numbers of Salmonellae than plain tetrathionate broth. The only non-lactose-fermenters recovered from novobiocin-tetrathionate broth, however, were Salmonellae, and growths were always heavier than those from tetrathionate broth.

Since it was shown in a separate experiment that small numbers of the *Salmonella enteritidis* strain used in experiment (f) survived 40 μg./ml. novobiocin

in tetrathionate broth the poor growth of this Salmonella was not due to a harmful effect of novobiocin.

Incorporation of Novobiocin in Desoxycholate-citrate-agar. — Desoxycholate-citrate-agar (D.C.A.) suffers from the disadvantage that it readily supports the growth from faeces of most strains of Proteus. It was therefore considered worth while to investigate the possibility of incorporating novobiocin in D.C.A. in such concentrations that Proteus would be inhibited and the medium would not be rendered more toxic for Salmonellae and Shigellae.

In order to test the ability of desoxycholate-citrate-agar to support the growth of the test organisms, 18-hour broth cultures were diluted 1/100, and one loopful was spread over a segment of a D.C.A. plate, which was then incubated for 18 hours. Of 14 strains of Proteus, nine grew well, four grew poorly, and one failed to grow. Good growth was obtained with 14 Shigella strains (*S. sonnei* six, *S. boydii* two, *S. flexneri* six) and five strains of Salmonellae, including one strain of *Salmonella typhi*.

Growth on Desoxycholate-citrate-agar Containing Different Concentrations of Novobiocin.— Desoxycholate-citrate-agar containing novobiocin, 100 μg./ml., was inoculated with dilute broth cultures. It was found that this medium, although completely inhibitory to all the Proteus strains tested, was also markedly inhibitory to the strains of Salmonellae and *Shigella sonnei*. Desoxycholate-citrate-agar plates were then prepared containing novobiocin in concentrations from 50 μg./ml. to 2 μg./ml. of medium and inoculated with dilute broth cultures of the following organisms (the number of strains tested is shown in brackets): *S. boydii* (2), *S. sonnei* (2), *S. flexneri* (6), *Salm. thompson* (1), *Salm. typhi* (1), *P. vulgaris* (1), *P. mirabilis* (2), *P. morganii* (1). Plates were incubated overnight.

The highest concentration of novobiocin in D.C.A. which did not affect the growth of the Shigellae and Salmonellae tested was 16 μg./ml. of medium, and this did not prevent the growth of three-quarters of the Proteus strains. Desoxycholate-citrate-agar containing 32 μg. novobiocin/ml. medium, whilst inhibiting the growth of half the strains of Proteus, was markedly inhibitory to one strain of *S. flexneri*, slightly inhibitory to one strain each of *S. sonnei* and *S. boydii*, and without effect upon growth of *Salm. thompson* and *Salm. typhi*, but 50 μg. novobiocin/ml. medium completely inhibited growth of all strains of Proteus and one strain of *Salm. typhi*.

Thus the lowest concentration of novobiocin tested in D.C.A. which effectively inhibited Proteus was also markedly inhibitory to Shigellae and *Salm. typhi*, so that the medium is unsatisfactory for routine use.

Proteus Suppression in Routine Faeces Cultures in Novobiocin-tetrathionate Broth.—During a three-month trial, in which 0.5 ml. of a suspension of each specimen of faeces examined was inoculated into both novobiocin-tetrathionate broth and tetrathionate broth, no Salmonellae were encountered. Novobiocin–tetrathionate broth effectively suppressed Proteus when the specimens contained insufficient numbers to give profuse growths on direct cultures.

Discussion

In a discussion of the comparative merits of Salmonella enrichment media Rappaport *et al.* (1956) assigned to their new medium a " valency coefficient " (Lang, 1952, 1954), showing it to be superior to the medium of Bierbrauer: these results were based on tests in which only one strain of each of two organisms was used.

From the experiments (a) and (c) with tetrathionate broth (Table II), it will be seen that when two different strains of Proteus were tested with a single strain of Salmonella markedly different valency coefficients were obtained.

The term " valency coefficient " is thus shown to be misleading, since its value will vary according to the strains of organisms tested. The conclusions of Rappaport *et al.* concerning the comparative selectivity of their medium may therefore be erroneous.

Although the value of enrichment media for isolating members of the Salmonella group is established, there is little agreement upon the relative merits of the many types and their modifications which have been described. Experimental work has shown that no one medium may be suitable for all members of this large genus and that the maximum number of Salmonella isolations is made when several media are used simultaneously (Collard and Unwin, 1958).

It seems likely that the addition of novobiocin would improve the selectivity of all liquid media for Salmonellae, but controlled trials in each medium would be required, as the combination of novobiocin with different selective agents might inhibit Salmonellae, as was found in the tests described above with D.C.A. As tetrathionate broth is one of the enrichment media commonly used in this country it was chosen for trial in the first instance.

A comparison of the proposed medium with other enrichment media for the isolation of Salmonellae from faeces of infected patients was not possible, owing to the infrequency of Salmonella infections in this area.

Summary

Of the non-lactose-fermenting Gram-negative bacilli encountered in faeces, members of the Proteus group are the most sensitive and Salmonellae are amongst the most resistant to novobiocin.

Tetrathionate broth containing 40 μg. novobiocin/ml. medium has proved superior to plain tetrathionate broth as an enrichment medium for the isolation of Salmonellae from faeces artificially infected with known numbers of Salmonellae and Proteus.

Novobiocin at a concentration of 40 μg./ml. medium in tetrathionate broth remained stable for at least 48 hours at 37° C., and for one month at room temperature. Results of stability tests in other enrichment media are reported.

The criteria used for the quantitative comparison of enrichment media are discussed, and the term "valency coefficient," as used by Rappaport *et al.*, is shown to be misleading.

Desoxycholate-citrate-agar containing novobiocin in a concentration sufficiently high to inhibit Proteus is also inhibitory to Shigellae and *Salm. typhi* and is therefore not useful for the direct culture of faeces.

To Professor Wilson Smith, F.R.S., and Dr. E. J. Stokes I am indebted for helpful criticism and guidance during the course of this investigation and in the presentation of the results. I am also grateful to Dr. Joan Taylor and Dr. K. P. Carpenter for providing Salmonella and Shigella strains, and to Dr. A. R. H. Hicks, of Upjohn of England Ltd., for an ample supply of novobiocin.

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TECHNICAL METHODS

A Dye-resin Test for Achlorhydria

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(RECEIVED FOR PUBLICATION APRIL 20, 1959)

The use of resins as a basis for "tubeless test meals" has made the unpleasant experience of aspiration of gastric contents unnecessary in many cases. A substance incorporated in the resin is released in the stomach only in the presence of free hydrochloric acid. This substance is then absorbed into the blood and excreted in the urine. Originally quinine was used in the test, and estimated fluorimetrically in the urine (Segal, Miller, and Morton, 1950; Harkness and Durant, 1953). Later, methylene blue was used instead of quinine, as it is more easily detected (Goldbloom, Feinstein, and Eiber, 1955; Segal, Miller, and Plumb, 1955).

Although different modifications describe varying degrees of quantitative exactness, the method is essentially a qualitative one. If no dye appears in the urine the patient is presumed to have achlorhydria. No measure of the level of gastric acidity is shown by the amount of dye in the urine. As a simple qualitative test for achlorhydria the dye-resin test can be substituted for a test-meal in many cases. When prepared in the laboratory the cost per test is between two and three pence instead of 4s., which is the cost of the proprietary preparation.

Method

The following requirements are sufficient for 40 to 50 tests.

(1) Amberlite resin I R C 50 (H), B.D.H., 100 g.

(2) Azure 1, B.D.H., 5 g.

(3) Two-litre conical flask with side-arms.

The Dye Resin.—The dye is completely dissolved in a litre of distilled water with stirring. The solution is poured into a 2-litre conical flask fitted with a cork containing a piece of glass tubing reaching nearly to the bottom of the flask. The resin granules are added, and the contents of the flask well stirred, and then agitated overnight by attaching the side arm to a vacuum pump. Care must be taken that the flow of air is not so fast that the solution bubbles into the side-arm. On the following day the resin is allowed to settle, and the supernatant sucked off. About 800 ml. of distilled water is added, the solution well mixed, then allowed to stand, and the supernatant again sucked off. This procedure is repeated six more times during the day, and then a final addition of water is mixed in and allowed to stand overnight. A completely colourless supernatant is not achieved, but the colour should be very pale. The granules are then filtered off by means of a large funnel and No. 1 Whatman filter paper, using more distilled water as required to wash all the granules out of the flask. The filter paper containing the granules is placed in a dish and dried in an oven at approximately 50° C. for two days. A paraffin oven at 50° C. as used in any histological department is suitable. Alternatively an incubator set at 37° C. for four days can be used. After drying, the navy-blue granules should lose no colour when shaken in water, but on shaking for a minute in N/10 hydrochloric acid should make the liquid pale blue. The dried granules can be placed in a screw-top jar, and dispensed as required. Although these quantities should theoretically produce sufficient for 50 tests, some granules are inevitably lost during the process, and elaborate precautions against this loss make the preparation tedious.

The Test

The patient receives no breakfast, but drinks of water are allowed. The early morning specimen of urine is discarded, and an hour later a specimen of urine is collected (specimen 1). Immediately after this 0.25 mg. of histamine is injected intramuscularly, and immediately after this 2 g. of dye resin is given in a glass of water, stirring well, so that all the granules are swallowed. Two hours later the urine is collected (specimen 2). Both specimens of urine are sent to the laboratory.

Results

Specimen 1 is used as a control, and compared with the colour of specimen 2. If the latter is obviously tinged green or blue there is free acid in the gastric juice. The shade varies not only with the amount of dye, but with the concentration of normal urinary pigments. If there is no obvious difference between the two specimens the reaction of both should be adjusted roughly to *p*H 3 with a drop of 20% HCl, and boiled. After cooling traces of dye may now be apparent. If there is still no difference between the two specimens it is assumed that the patient has achlorhydria. A quantitative report should not be given.

A trace of green may be present in the urine after four hours in persons with achlorhydria. Only the two-hour result, therefore, is significant. The patient should be warned that he may pass blue urine for three days after the test.

Discussion

This test is quick, cheap, simple, and painless. It is adequate in most cases for the diagnosis of achlorhydria. The usefulness of test meals for diagnosing any other gastric abnormality is questionable.

The term "achlorhydria" is usually taken to mean that the gastric juice has a *p*H of more than 3, as shown by Topfer's reagent or thymol blue. It denotes a relative "an"-acidity rather than a complete absence of hydrochloric acid.

The results of the dye-resin test can be varied by altering the quantity of dye and resin and the time of urine collection. A standardized procedure, therefore, is necessary.

The conditions which may give misleading results in resin tests have been described by Harkness and Durant (1953). They include the administration in the two days before the test of such ions as aluminium, magnesium, calcium, barium, iron, and kaolin that may release dye from the resin. Pathological states which may interfere with excretion of the dye include pyloric stenosis, intestinal malabsorption, and diseases of the urinary tract.

The commercial preparation of the dye resin ("diagnex," Squibb) substitutes 500 mg. of caffeine sodium benzoate for histamine. Although caffeine is a powerful gastric stimulant, the meaning of achlorhydria is generally taken to be the absence of free acid after histamine stimulation. It is therefore preferable to use histamine instead of caffeine.

Summary

A simple and cheap method of preparing and using a dye-resin compound as a test of achlorhydria is described.

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Beginning with the issue of the *Journal of Clinical Pathology* of January, 1960, summaries of papers will be replaced by abstracts written by the author. These abstracts will precede the paper, and will be fuller than most summaries. For the guidance of contributors the following suggestions may be useful:

The abstract should always include (1) a statement of the purpose for which the investigation or review was undertaken; (2) the selection of material used; (3) the methods, procedures, or treatment employed; (4) the results; (5) discussion.—EDITOR.

A Jig for the Agla Micrometer Syringe for Automatic Quantitative Applications in Paper Electrophoresis

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(RECEIVED FOR PUBLICATION MAY 16, 1959)

The device described here is designed to achieve quantitative applications combined with an even distribution of material by means of continuous automatic expulsion from the micrometer syringe as the needle moves across the strip. The method is suitable for use with electrophoresis tanks of the horizontal type with the strips already in position. The inexpensive jig can be made quite easily from "perspex."

An "agla" syringe with a micrometer attachment is mounted by means of Terry clips (A in Fig. 1) on a laterally moving platform (B). When in position, the machined handgrip of the micrometer rests firmly against a rubber strip (C) supported on a fixed upright. When the locking screw (D) is released, movement of the platform causes the micrometer screw to turn. The platform is moved by hand and is limited by a removable check pin (E) capable of insertion at intervals corresponding to the width of the strip. The base is in two parts (F and G) sliding on each other when the locking lever (H) is released to allow lining up of the needle point with the application line on the strip. A long needle ("record" No. 20), filed level at the end, is attached to the syringe and bent at right angles to reach the strip through a narrow opening in the tank. The height of the needle is finally adjusted by means of the screw (I) operating against the flap (J). The front end of the base-plate (G) rests against a bevelled "perspex" bar on the lid of the tank fixed in such a position as to allow the needle to pass through the opening and at the same time permit both tilting and lateral movement of the jig on the lid. On the under surface of (G) is a strip of rubber (K) so that pressure of the hand causes the base-plate to grip the lid during movements of the platform (B).

The handgrip of the micrometer has a circumference of exactly 2 in., so that lateral movement of 1 in. results in the expulsion of 0.005 ml. of fluid. Obviously, the maximum number of consecutive applications depends on the width of the strip and the jig respectively. The apparatus is reset for further applications by locking the micrometer and pulling the platform across with slight upward pressure to clear the rubber strip (C).

Fig. 2 shows the complete apparatus with the platform (B) in mid position.

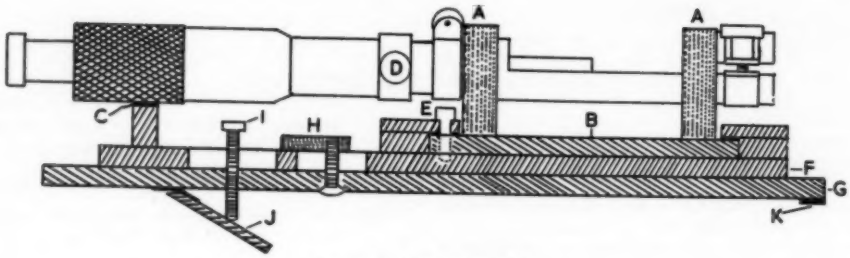

FIG. 1.—Mid-line section of jig.

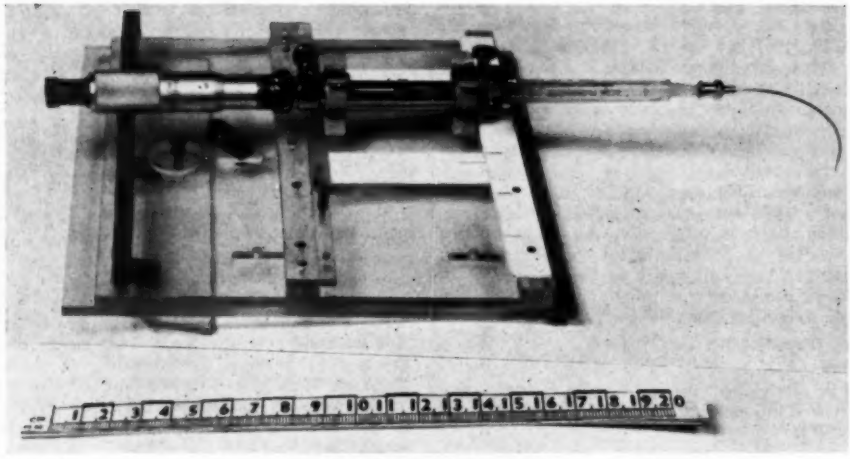

FIG. 2.—The complete apparatus.

Operating Procedure

(1) The syringe is charged, clamped in the micrometer attachment, and fitted into the jig.

(2) The platform (B) is now moved to check delivery from the needle tip, each drop being gently touched off with the finger.

(3) The jig is now placed in position on the lid so that the needle lies beside the strip. The tip of the needle is lined up with the application line and its height set just below the surface of the strip.

(4) A folded piece of filter paper is held for a few seconds on the application line to soak up excess buffer.

(5) With the micrometer released and the check pin one position ahead, the whole jig is lifted slightly to elevate the needle tip and to place it on the application line at the extreme edge of the strip.

(6) With firm pressure on the base-plate, the platform (B) is now moved *slowly* across until it reaches the check pin.

(7) By releasing the pressure on the base-plate the jig can be moved sideways to carry the needle clear of the strip and the application is completed.

To obtain satisfactory results the following points must be borne in mind: (*a*) Elimination of air bubbles from the syringe; (*b*) a flat, rigid tank lid on which to rest the jig; (*c*) removal of excess buffer from the application line; (*d*) careful adjustment of needle height; (*e*) slow movement of the needle across the strip. With Whatman 3 mm. paper this should take about 20 seconds per inch. Faster movement leads to pooling. When using cellulose acetate membrane better results are obtained if, at the end of the application, the needle is moved backwards and forwards across the strip several times. (*f*) The use of a "polythene" tip on the needle may be of value when using cellulose acetate membrane but was not found to be in any way advantageous for applications to filter paper.

An Automatic Device for Controlling Hot Air Ovens

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From the Department of Haematology, Royal Perth Hospital, Perth, Western Australia

(RECEIVED FOR PUBLICATION FEBRUARY 21, 1959)

The automatic device to be described offers the following advantages when fitted to hot air sterilizers:

The oven may be switched on and left, when the temperature will rise to 160° C. or any other preset temperature. The temperature will be maintained for any preset length of time up to one hour, and after this the unit will switch off the power. The complete cycle is automatic so that the need for an operator to watch the temperature and the clock is eliminated. The oven may be switched on just before the laboratory closes in the evening; it will then complete the warming up and sterilizing cycle automatically.

The device (Fig. 1) consists of a relay with two pairs of contacts. The lower contacts are closed except when the relay is energized; the upper contacts are used to complete the energizing circuit. The relay is wired to the hot air oven as shown, one wire going to the power and the other to the sensitive side of the thermostat. The clock section of a Venner process timer (model P.T.4) is connected to the power via the lower relay contacts and the process timer switch section is connected between the main supply and the oven. Controls include stop and start buttons and an automatic/manual switch. A pilot light indicates when the relay is energized.

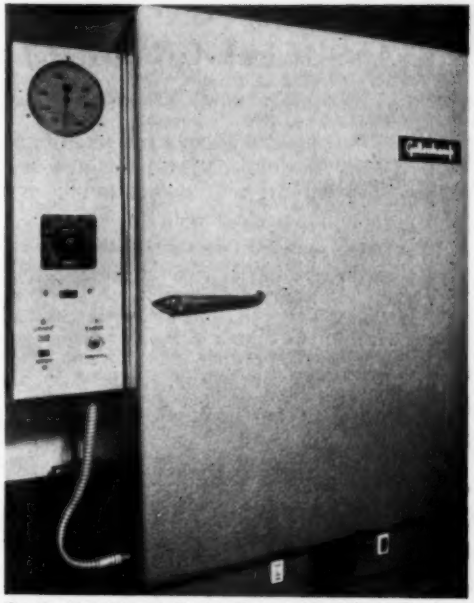
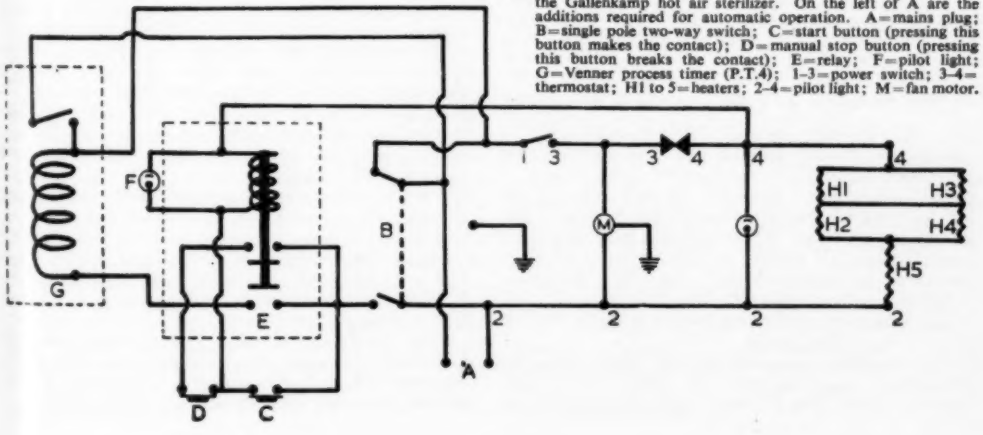


FIG. 2.—Complete unit fitted to the hot air sterilizer. The thermometer is not part of the circuit.

The power is switched on and the "start" and time switch buttons are pressed. The start button sets the relay with the upper contacts closed (E, Fig. 1). The oven temperature will now rise until the thermostat is activated and opens the circuit to the heaters and the relay. Opening the relay circuit will complete the timer circuit. The timer may be set for any period up to one hour, after which it will switch off the current.

FIG. 1.—The circuit to the right of A is that already incorporated in the Gallenkamp hot air sterilizer. On the left of A are the additions required for automatic operation. A=mains plug; B=single pole two-way switch; C=start button (pressing this button makes the contact); D=manual stop button (pressing this button breaks the contact); E=relay; F=pilot light; G=Venner process timer (P.T.4); 1-3=power switch; 3-4=thermostat; H1 to 5=heaters; 2-4=pilot light; M=fan motor.



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ASSOCIATION OF CLINICAL PATHOLOGISTS: 63rd GENERAL MEETING

The 63rd general meeting was held at the Royal College of Surgeons, London, from October 1–3, 1959. The Presidential address is published in full in this issue, and summaries of the other papers follow. The last day was devoted to a joint meeting with the Association of Clinical Biochemists.

Laboratory Control of Obstetrical Defibrination

A. A. SHARP (Oxford) said that experience in the investigation of obstetrical emergencies with suspected defibrination led to the adoption of four specific tests for the diagnosis and control of therapy. These tests are: (1) Whole blood clotting time (Lee and White, 1913); (2) thrombin time (Sharp, Howie, Biggs, and Methuen, 1958); (3) thrombin titre (Sharp *et al.*, 1958); (4) ammonium sulphate method for rapid estimation of fibrinogen (Biggs and Macfarlane, 1957).

In the past four years 33 cases have been examined and in 10 a defect has been demonstrated.

Six cases were described which illustrate the efficacy of these tests.

The results obtained showed that they are able to provide a rapid and reliable means of establishing the existence of fibrinogen deficiency and a measure of the effect of therapy.

These tests when compared with the more conventional biochemical methods of estimating fibrinogen suggest that in the early stages of obstetrical defibrination there may be qualitative changes in the reactivity of fibrinogen to thrombin before there is an actual depletion of circulating fibrinogen such as is encountered in severe cases.

Concentrated fibrinogen has proved to be effective in the treatment of these cases, and it has been given where the coagulation defect has been associated with a definite haemorrhagic state or where there has been a severe defect as measured by these tests. Such therapy has been instituted before labour has started with no detrimental effect, and in none of the cases has there been any evidence of continuing defibrination during therapy.

The Prevention of Cross-infection in Urology

K. B. LINTON, ASHTON MILLER, and W. A. GILLESPIE (Bristol) studied the epidemiology and prevention of urinary infection in three groups of patients whose urine was sterile initially and who did not have suprapubic drainage of the bladder. The groups were: (*a*) Transurethral and retropubic prostatectomy, (*b*) simple catheter drainage of acute retention, (*c*) gynaecological operations involving continuous post-operative bladder drainage.

The incidence of infection was high—over 70% in each of the three groups.

The causative organisms usually came from the environment by cross-infection. They were Gram-negative bacilli, *Staph. aureus* and streptococci, and mostly were abnormally resistant to antibiotics. In a minority, the causative organisms came from the patient's own body. Most infections lasted for several months. A few had serious consequences such as septicaemia.

Controlled trials showed that infection in males could usually be prevented by excluding organisms from the urethra and indwelling catheter. The incidence of infection after prostatectomy was reduced from 74% to 6% by a combination of precautions: pre-operative disinfection of the urethra, improved method of disinfecting cystoscopes, and a closed aseptic method of draining and irrigating the bladder.

In acute retention, only 10% of patients on closed drainage developed infection compared with 73% on open drainage. It was essential to prevent air bubbles from ascending the tube.

In the gynaecological patients, closed drainage brought some improvement, but further measures must be investigated.

Interpretation of Antibiotic Inhibition Zones in Mixed and Pure Cultures

E. JOAN STOKES (University College Hospital, London) said that there were two schools of thought about disc sensitivity tests: one that they should be done only on heavily seeded pure cultures, the other that they should be made on the original material and repeated when necessary.

Evidence was presented to show why the second method is preferred. With sensitive organisms there is no insurmountable difficulty in reading zones in mixed culture, and with partly or fully resistant organisms, whatever simple method is employed, it is impossible to be sure that the drug will be useless in treatment without skilled assessment. In the first instance confusion is avoided if the strain is reported as "not fully sensitive."

A method of testing primary cultures by comparison of zones with a known sensitive control was described. In a series of tests on 910 specimens, a suitable drug could be recommended after overnight incubation in 73% of cases and no further tests were necessary. In 93% of cases valuable information was obtained by the primary culture.

Other important advantages of a primary test are that resistant variants are recognized when they would otherwise be missed; pathogens overgrown on the diagnostic culture may be selected by the drugs, and

the drug sensitivity pattern is of value in indicating tests for identification and in giving preliminary reports.

"Collecting Capillary Blood"

R. H. WILKINSON (Hospital for Sick Children, Great Ormond Street, London) described a film (Kodachrome, 16 mm., silent, 200 ft.) devised to teach medical and technical staff how to obtain satisfactory and adequate specimens of blood from infants, children, and adults by skin puncture. The blood can be examined by micro-methods and the results are of particular importance in the diagnosis and therapy of children's diseases.* The film was directed and photographed by Derek Martin and David Floyd (Department of Medical Illustration) and demonstrated by Gillian Reynolds and Ann Cummins (Department of Chemical Pathology) at The Hospital for Sick Children, Great Ormond Street, London.

Changes in the Human Hypothalamus after Pituitary Stalk Section or Hypophysectomy

E. BECK and P. M. DANIEL (Institute of Psychiatry, Maudsley Hospital) reported nine selected cases out of a series of 21 patients, who had undergone the operations of hypophysectomy or pituitary stalk section for the relief of cancer and whose ages ranged from 39 to 70 and post-operative survival times from three weeks to 29 months. In all an impenetrable barrier had been placed between the hypothalamus and the pituitary fossa. The hypothalamus was cut serially and treated with a variety of staining methods. In the cases surviving briefly a venous infarct containing an accumulation of amorphous neurosecretory material was seen occupying the centre of the proximal stump of the pituitary stalk. The infarct had destroyed the supra-optico-hypophysial tract, the nerve fibres of which ended in retraction bulbs above the infarct. Between two and 26 months after operation the stump had enlarged and become reorganized and reinnervated, large numbers of nerve fibres being present. A prominent reticulin network throughout the stump, not normally seen, suggested that an infarct had originally been present, as did the presence of haemosiderin-containing macrophages. Many of the regenerated nerve fibres appeared as beaded fibres in Gomori chrome-alum-haematoxylin preparations, thus indicating that they were carrying neurosecretory substance. This substance was also seen in the walls of blood vessels and as Herring corpuscles of varying sizes. The structure of this reorganized and enlarged proximal stump was reminiscent of the normal posterior lobe of the pituitary gland. The pars tuberalis seemed to have hypertrophied and contained thick bundles of nerve fibres which were not seen in normal cases. These bundles did not contain Gomori-positive fibres. However, in a considerable series of experimental animals with pituitary stalk section examined by the authors, Gomori-positive fibres were prominent in similar nerve bundles ramifying amongst the glandular cells of the much hyphertrophied pars tuberalis.

The supra-optic and paraventricular nuclei which supply the bulk of the nerve fibres to the posterior lobe of the pituitary showed some nerve cell loss after three weeks and severe cell loss accompanied by dense gliosis after longer survival, a finding contrasting with that of Sloper and Adams (1956) of no gliosis in their two cases of long survival. Some of the surviving nerve cells and fibres always contained Gomori-positive granules. The patient who survived for 26 months had hardly any large nerve cells left in these nuclei. The cells of the tuberal nuclei, which give rise to the tubero-hypophysial tract, showed a very striking appearance, being much swollen, having a curious foamy cytoplasm and eccentricity of the nucleus even greater than that normally seen in neurosecretory nerve cells.

The appearances found suggest that an attempt is being made in the proximal stump of the pituitary stalk to form a reinnervated structure resembling the posterior lobe of the pituitary gland.

Renal Biopsy: Some Observations on the Histology of the Glomerulus

M. S. R. HUTT, H. DE WARDENER (London), and S. C. SOMMERS (Boston, U.S.A.) said that the most significant advances in renal pathology in the last few years had been made by renal biopsy and electron microscopy. Renal biopsy has the great advantage of enabling the pathologist to study the earliest lesions of a disease process; electron microscopy has shown the fine structure of the normal glomerulus as well as revealing lesions not visible by ordinary light microscopy.

In handling renal biopsy specimens for routine light microscopy the specimen should be fixed immediately in Suza's or Zenker's fluid, and after processing in the usual way sections should be cut at 2 to 4 μ. Mowry's modification of the colloidal iron-P.A.S. stain is of particular value in the examination of glomerular structure in view of its specific staining of both the basement membrane and the borders of the epithelial cell cytoplasm. Using these techniques the sections can be examined using a 1/12 oil immersion lens, and this may reveal abnormalities not apparent with routine techniques.

The application of these techniques to renal biopsy specimens from cases of glomerulonephritis with proteinuria was illustrated. In several of the cases studied the striking abnormality was swelling of the epithelial cells of the tuft, and in the colloidal iron-P.A.S.-stained sections these cells frequently were separated from the basement membrane, which showed varying degrees of thickening. These appearances are similar to those described in two cases of glomerulonephritis by Movat and McGregor, who demonstrated by electron and light microscopy swelling and vacuolation of epithelial cells with deposition of protein between these cells and the basement membrane.

*Wilkinson, R. H., *Chemical Micromethods in Clinical Medicine*, 1959. Thomas, Springfield.

Retro-corneal "Membrane" after Keratoplasty

A. SACHS (East Grinstead) described a complication of full-thickness keratoplasty which leads to corneal opacification, namely, the formation of a membrane deep to the graft.

The early clinical manifestations are a 15 to 25% increase in the thickness of the graft, epithelial and stromal oedema, and wrinkling of Descemet's membrane. Vascularization may also be present in some cases.

The histopathological findings show that there is epithelial and stromal oedema and that a connective tissue membrane is present on the deep surface of Descemet's membrane. The posterior surface of the membrane is covered by endothelium. Descemet's membrane is wrinkled and there is a gap between donor and host parts. The unattached end of the graft Descemet's membrane is coiled like a whip lash.

The probable cause of this condition is defective wound healing and not the result of a homograft reaction.

Pneumoconiosis due to Mica

A. MEIKLEJOHN (University of Glasgow) said that 75% of the world output of mica is produced in the province of Bihar, India. The deposits occur in silicious strata, and the occurrence of silicosis among the miners is well established. So far there is little experience of pulmonary disease among workmen employed in processing pure mica; indeed the number of persons at risk is small. After the discovery of Dr. Osborn's case (see below) all the other workmen employed at the same factory were surveyed. Four further cases were diagnosed, all apparently in the early stage. The clinical and radiographic features were related to asbestosis rather than to silicosis or coalworkers' pneumoconiosis. A programme of strict engineering and medical control in co-operation with the factory inspectorate was immediately established by the company, and is being actively maintained to safeguard the health of all employees.

Patterns of Certification of Coalworkers' Pneumoconiosis in Great Britain

A. MEIKLEJOHN (University of Glasgow) said that many excellent surveys on the prevalence of pneumoconiosis among coal-miners had been made by research teams in separate coalfields in Great Britain. These, however, even cumulatively, do not provide a comprehensive view of the present situation in the country as a whole. This information may ultimately be provided by the 25 Pits Scheme under the National Coal Board and the periodical radiographic examination of all coal-miners, which began at the end of 1958. Meanwhile the Digest of Pneumoconiosis Statistics published by the Ministry of Power beginning in 1951, which provides data of official certification under the National Insurance (Industrial Injuries) Acts, is the only available index. It is recognized that such diagnosed cases are subject to many influences and so provide only a crude measure of prevalence, precluding narrow comparisons between divisions and areas. The analyses, as it happens, reveal striking differences and contrasts in application rates, certification rates, age incidence, and degree of disablement. These are examined in a series of charts and tables covering the years 1951–7. The analyses reveal an extremely favourable situation in the East Midlands Division, which included the Nottinghamshire area.

Antibiotics from Coal

W. D. EVANS (Nottingham) said that carboniferous coals have yielded antibiotic remains of the microbiological decay of plants. Collectively called "vitricin," they seemed to exhibit prophylactic control over the incidence of coal-miners' pneumoconiosis. Dusts rich in vitricin are found in mines of low incidence and vice versa. Over 200 tests *in vitro* indicated that vitricin was bacteriostatic to *Mycobacterium phlei, Staphylococcus aureus, Pseudomonas pyocyanea,* and the tubercle bacillus $H_{37}R_V$. That vitricin consisted of phenolic bacteriocides was eliminated at the outset. A guinea-pig test *in vivo* indicated low toxicity and significant inhibitions of the tubercle $H_{37}R_V$. The acidic content of vitricin proved toxic. The basic constituents proved inactive, but the neutral components strongly inhibited *B. subtilis, M. phlei,* and *Staph. aureus.* Sixteen neutral bacteriostatic constituents of vitricin have been isolated. Serial dilution tests of vitricin against *M. phlei* have shown activities up to 1 part in 19,000,000, but these are often suppressed in the presence of serum.

Vitricin also exhibits fungistatic properties and proved active against *Aspergillus niger, Aspergillus terreus, Myrothecium verrucaria, Verticillium alboatrum, Endoconidiophora fagacearum, Cerstostomella ulmi, Glomerella cingulate,* and *Colletotrichum trifolii.*

Investigation of the biological properties of vitricin has been limited by the small yield obtained from coal. Small samples, not exceeding 1 cwt. of coal, have been processed, but these were too small for toxicity tests. Since these antibiotics have been produced during the formation of coal and preserved under exceptional conditions, their evaluation might bring into existence organic substances which hitherto have not found a place in the chemotherapy of disease.

[This paper will be published in full in a later issue.—ED.]

Pneumoconiosis due to Mica

G. R. OSBORN (Derby) reported the following case: W. W., aged 47, worked with finely ground mica for eight years. In February, 1957, routine mass radiography showed pneumoconiosis. His first symptoms developed in April, when he became short of breath on exertion. In May he suffered from abdominal pain which was not relieved by anything; his urine became dark at this time. Dyspnoea increased, he became

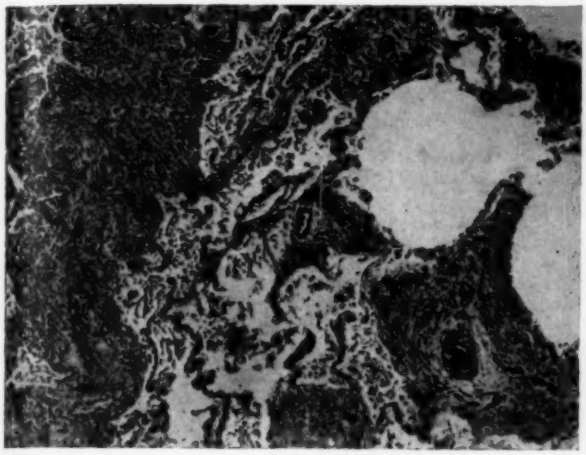

FIG. 1

FIG. 2

FIG. 1.—Low-power view (×40) of the fibrosis induced in the lungs by mica.

FIG. 2.—Birefringent mica flakes in the same field as Fig. 1 revealed by polarized light.

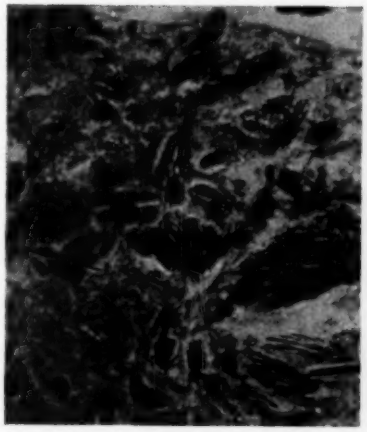

FIG. 3

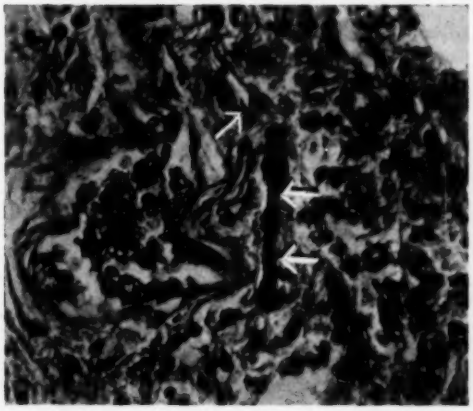

FIG. 4

FIG. 3.—High-power view (×960) of reaction to mica by macrophages and fibrous tissue.

FIG. 4.—Asbestosis body (two arrows). An early stage in the formation of an asbestosis body is shown by the single arrow; a knob has formed around a mica needle which is still optically active. Numerous mica flakes can be recognized throughout this section (×350).

apprehensive, and died on June 4 in a uraemic state. The blood urea level rose from 164 mg./100 ml. on May 16 to 270 mg./100 ml. on the day before he died. The urine contained much albumin and many casts with red and white blood cells.

Necropsy showed the renal lesion responsible for his death to be polyarteritis nodosa, but its relation to the mica pneumoconiosis was not clear. The heart showed hypertrophy of the left ventricle. The trachea and bronchi contained blood-stained froth and mucus. Both lungs were firm like rubber, they were greyish-brown, and showed diffuse fibrosis with small emphysematous patches. The right lung weighed 40 oz. (1,134 g.) and the left 38 oz. (1,077 g.). Dr. Nagelschmidt, of the Mines Research Department in Sheffield, showed that 15% of the dried weight of the lungs was mica (muscovite).

Microscopic examination of the lungs showed a large amount of mica, nearly all in the form of birefringent needles (not many plates). It had excited a profuse macrophage reaction and fibrosis unlike that of silicosis. Macrophages with ingested mica flakes

more or less filled most of the alveoli. Alveolar walls were greatly thickened by these macrophages and by fibrosis. Foreign body giant cells were fairly numerous throughout. An unexpected finding was asbestosis bodies in all stages of development. The earliest stages in the development of these bodies is around a mica flake that cannot be recognized in the later stages when the optical properties are lost. The amount of iron revealed by the Prussian blue reaction was also unexpected. There are three possible sources of this: (1) Endogenous, especially in the formation of asbestosis bodies; (2) red mud in small amount, containing Fe_2O_3, is mixed with the mica when received at the factory; (3) muscovite mica is not iron-free, as most books state, but contains upwards of 5% iron in the crystal lattice, but would not be likely to give a Prussian blue reaction unless it was broken down in the body. The tracheobronchial lymph nodes contained an appreciable amount of mica; it was not demonstrated beyond them.

Auto-immunity in Thyroid Disease

I. M. ROITT and D. DONIACH (Middlesex Hospital, London) said that the principal auto-antigens involved in Hashimoto's thyroiditis are thyroglobulin in the acinar colloid and an intracellular constituent particularly abundant in thyrotoxic glands. Antibodies to thyroglobulin may be demonstrated in 90% of Hashimoto patients using precipitation in gel or tanned cell agglutination techniques. The latter test has been simplified for routine clinical use by the development of a stable preparation of formalized thyroglobulin-coated sheep red cells.

The intracellular antigen is localized to the "microsome" fraction of thyroid epithelial cells, and gives rise to complement-fixing antibodies which are present in over 90% of Hashimoto patients. These two immune systems are completely independent, and some patients have only one or the other of the antibodies. The separate nature of these can be clearly demonstrated by the Coons' fluorescent antibody technique. Thyroglobulin antibodies stain the colloid in alcohol-fixed sections whereas complement-fixing antibodies stain the cytoplasm of the acinar cells in frozen unfixed sections.

Evidence has been obtained that certain Hashimoto patients who have raised sero-flocculation tests but give negative results by tanned red cell and complement-fixing tests have antibodies directed against a colloid constituent other than thyroglobulin, demonstrable by the Coons' technique.

Another abnormal serum constituent which may be an auto-antibody has been detected in Hashimoto sera by its cytotoxic effect on thyroid cells in tissue culture. This cytotoxic factor is inactivated by heating to 56° for 30 minutes and has only been shown to kill trypsinized thyroid cells cultured for up to 36 hours.

In the majority of thyroiditis patients, auto-immune phenomena are confined to the thyroid gland, but about 10–15% of cases show more widespread disturbances of immunological reactions and either give positive complement fixation with saline extracts of other human organs or nuclear fluorescent staining connected with lupus erythematosus. Very occasionally patients have both thyroiditis and evidence of systemic lupus with positive L.E. cells.

It is considered that the lymphadenoid lesions are a consequence of auto-immunization. Although focal lesions are frequent in all types of goitres, particularly in thyrotoxicosis, progressive destruction of the thyroid gland is relatively infrequent. The presence of the circulating antibodies so far characterized seems inadequate to account for progression of the lesions in the Hashimoto gland, although the role and nature of the agent cytotoxic to tissue cultures have yet to be elucidated. In the experimental auto-allergic diseases the view is current that hypersensitivity of the delayed type is responsible for the histological damage, but so far the evidence in the human thyroid disease is inconclusive. It is of interest in this connexion that the lymphocytes of the Hashimoto gland exhibit unusual behaviour in tissue culture and appear to be attracted to the thyroid cells and even seem to enter into their cytoplasm.

Observations on Thyroid Auto-antibodies

J. R. ANDERSON, R. B. GOUDIE, and KATHLEEN G. GRAY (Glasgow) said that complement-fixing antibodies, reacting with saline extracts of thyrotoxic thyroid tissue, are found in 90% of cases of Hashimoto's disease, in over 50% of cases of thyrotoxicosis and primary myxoedema, and in 7% of hospital patients with no clinical evidence of thyroid disease. In the latter group a positive complement-fixing test is associated with mild focal thyroiditis; the presence of areas of Askanazy cell change and round-cell infiltration in the thyroids of these patients appears to be the result of auto-immunization. The common occurrence of complement-fixing antibodies in various thyroid diseases makes the test of little diagnostic value.

The precipitin test for circulating antithyroglobulin is positive only in patients with clinically significant thyroiditis (Hashimoto's disease, myxoedema, or severe focal thyroiditis complicating thyrotoxicosis or carcinoma of thyroid). Surgical treatment should only be undertaken in these patients when there is strong suspicion of carcinoma or when medical treatment fails.

Methods for the Estimation of Cholinesterases

W. N. ALDRIDGE (M.R.C., Carshalton) said that the cholinesterases are not specific for the hydrolysis of choline esters and will hydrolyse many non-choline esters. In this sense the cholinesterases are less specific than aliesterases and lipases which do not hydrolyse choline esters. For this reason only choline esters may safely be used as substrates for the assay of the cholinesterases. Two types of cholinesterase are known. One is present in erythrocytes and nervous tissue (true, aceto-), and

hydrolyses acetylcholine faster than the higher homologues. The other type of cholinesterase is present in plasma, pancreas, and other tissues, and hydrolyses the higher homologues faster than acetylcholine. These two types may be separately determined using acetyl β-methylcholine and *n*-butyrylcholine respectively for true and pseudo cholinesterase. The methods (with one exception) depend upon the measurement of acid produced during the hydrolysis. The manometric technique measuring CO_2 liberation from bicarbonate is the only method which fulfils all requirements of giving rate measurements, and is unaffected by protein content and colour of the enzyme preparations. Other methods based upon change of *p*H are extremely useful and may with certain tissues be used for the assay routinely of a large number of specimens. One colorimetric method has been published using β-carbonaphthoxycholine iodide as substrate. As with all enzyme assays, the conditions must be rigidly controlled. Provided necessary precautions are taken, the methods will give reliable results, but not all are of general application to all tissues.

Clinical Applications of Cholinesterase Estimations

R. H. S. THOMPSON (Guy's Hospital, London) said that the most extensively investigated condition in which the estimation of serum cholinesterase levels has been of real value is in patients with jaundice or with suspected liver damage. McArdle (1940) showed that this estimation can be usefully employed both as a measure of liver function and as a test in differentiating jaundice of hepatic and obstructive origin, the levels being very considerably reduced in hepatic jaundice and in hepatitis and cirrhosis, but hardly affected in obstructive jaundice.

Low levels are also found in malnutrition (Hutchinson, McCance, and Widdowson, 1951), the effect of which on the serum cholinesterase level is more pronounced than on the level of total serum proteins.

As is to be expected, intoxication by compounds belonging to the organo-phosphorus group of anticholinesterases can also cause a profound lowering of serum cholinesterase activity. Blood cholinesterase estimations can therefore be of value in detecting minor and often symptomless degrees of early intoxication in workers exposed to these reagents (Barnes and Davies, 1951).

The level of the serum cholinesterase is also of practical importance in connexion with sensitivity to succinylcholine. If the level of this enzyme is low, as in patients with liver disease, a prolonged apnoeic response may be obtained after the administration of the drug (Bourne, Collier, and Somers, 1952; Evans, Gray, Lehmann, and Silk, 1953). In this connexion it is important to remember the existence of a rare, genetically determined deficiency of this plasma enzyme (Lehmann and Ryan, 1956).

Raised levels of serum cholinesterase have been reported in diabetes mellitus. Thompson and Trounce (1956), however, have shown that high values in diabetics are only found when the patients are overweight, and that the level is similarly raised in obese subjects who are not diabetic.

High levels are also found in the nephrotic syndrome (Kunkel and Ward, 1947) and in thyrotoxicosis (Antopol, Tuchman, and Schifrin, 1937; Thompson and Tickner, 1950).

Although the cholinesterase levels both in blood and in muscle are normal in myasthenia gravis, it has recently been reported that the level of the true cholinesterase in the C.S.F. is raised in myasthenic patients (Bernsohn, Boshes, and Possley, 1958). However, the number of patients studied was small, and the finding needs confirmation.

Low levels of pseudo cholinesterase in the skin were found by Magnus and Thompson (1956) in cholinogenic urticaria, a finding which accords with the conclusion of Grant, Bruce Pearson, and Comeau (1936) that the wealing is due to an abnormal overreaction of the skin to the normal amount of acetylcholine released at nerve-endings in the skin.

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The Chemotherapy of Poisoning by Organophosphate Anticholinesterases

D. R. DAVIES (Ministry of Supply, Chemical Defence Experimental Establishment, Porton) reported that antidotes for organophosphate poisoning had been developed by repairing the biochemical lesion which is the primary cause of death. The organophosphates are lethal because they inactivate cholinesterase by phosphorylating the enzyme's active centre. Two rational lines of treatment are (*a*) to find some compound which could be phosphorylated as rapidly as the enzyme and which if introduced into the body would protect the enzyme from inhibition by competing with it for the organophosphate, or (*b*) to find a compound which would restore the activity of the inhibited enzyme by dephosphorylating it. In both cases essentially the same type of compound is needed, namely, one with a high intrinsic reactivity with organophosphoryl compounds in (*a*) with the inhibitor itself or in (*b*) with the phosphorylated enzyme. Types of compounds with either or both of these properties are *o*-dihydroxybenzene derivatives, metal chelates, hydroxylamine,

hydroxamic acids, and oximes. Derivatives of hydroxyiminomethyl-N-methylpyridinium have been by far the most successful as reactivators of the inhibited enzyme and in the treatment of poisoned animals.

In conjunction with atropine 2 hydroxyimino-methyl-N-methylpyridinium (2 PAM) has proved very successful in treating both animals and man.

More recently oximes of $\alpha\omega$ diquaternary alkane salts have been developed and shown to be even more potent reactivators, but have not proved to be generally and proportionately effective *in vivo*.

Secretory Response of Gastro-pepsin, Mucoproteose, and Glandular Mucoprotein to Histamine as Well as Insulin Stimulations

J. SCHRAGER (Wigan) said that he was concerned with an attempt to correlate the secretory pattern of the gastric mucosa with histological findings. Two hundred and fifty-seven patients were investigated; of these, 93 were proved later to have a duodenal ulcer, 40 a gastric ulcer, 38 a carcinoma, and 86 patients showed negative radiological results, and were labelled by the physician as "gastritis."

The following tests were carried out. The uropepsinogen was estimated in three consecutive 24-hour specimens of urine. A fractional gastric analysis was carried out using histamine and intravenous insulin as stimulants; free acid, gastric pepsin, mucoprotein, and mucoproteose were estimated in every specimen. Also a barium meal report for each patient was obtained from the radiological department. About 120 patients were ultimately operated on. The operation specimen was sent to the laboratory and the mucosa studied thoroughly. Thus it was possible to compare the biochemical data with the histological findings and contrast them with the barium meal report. The patterns obtained fall into the following groups.

(1) A high uropepsinogen, a high gastric pepsin, high acid, and a high mucoprotein and normal mucoproteose are associated with a hypersecretory mucosa and usually found in patients suffering from duodenal ulcer.

(2) A low uropepsinogen but a brisk response of secretion of free acid, mucoprotein, and pepsin to histamine and insulin were found in patients with a hypersecretory mucosa but with a superimposed gastritis.

(3) Patients with gastric ulcers provided secretory patterns which varied greatly. All the patterns were seen. The main factor responsible for the varying changes noticed in the gastric secretion is the state of the gastric mucosa. The whole spectrum of changes associated with gastritis was encountered in the histological examination of the mucosa.

(4) Patients with gastric carcinoma show a distinctive pattern: no uropepsinogen, complete absence of pepsin, gastric mucoprotein, and a very high mucoproteose. Whenever the carcinoma is situated near the fundus there will be complete absence of gastric mucoprotein, but some pepsin and acid will be found. In all cases there is a very high mucoproteose secretion.

Colorimetric Estimation of Serum Isocitric Dehydrogenase

JOYCE L. BELL and D. N. BARON (Royal Free Hospital, London) reported the method set out below:

Estimation of serum isocitric dehydrogenase is a valuable measure of hepatocellular damage (Baron and Bell, 1959). The standard method of estimation is spectrophotometric (Wolfson and Williams-Ashman, 1957), and because of its disadvantages in routine work a colorimetric procedure has been devised.

The enzyme carries out the following reaction:

$$\text{Isocitrate} + \text{TPN} \xrightarrow[\text{Mn}^{++}]{\text{ICD}} \text{oxoglutarate} + \text{CO}_2 + \text{TPNH}$$

The oxoglutarate is measured as the alkaline phenylhydrazone.

Method

Trisodium isocitrate (0.05 M)	0.5 ml.
TPN (0.01 M)	0.1 ml.
Tris buffer; *p*H 7.4 (0.1 M)	0.5 ml.
Mn^{++} (0.03 M)	0.1 ml.
Serum	0.5 ml.
Incubate at 37° C. for 60 min., then add:	
E.D.T.A. (5.6 g./100 ml.)	1 ml.
2:4 dinitrophenylhydrazine (19.8 mg./100 ml. *N* HCl)	1 ml.

Allow to stand at room temperature for 20 min., then add:

NaOH (0.4 *N*)	10 ml.

Allow to stand for 15 min., then read at 390 mμ against water.

Each serum has a blank containing all reagents except TPN. A separate TPN blank (without serum) is put through with each batch.

Standards containing 35 μg. and 70 μg. oxoglutarate per tube (+Mn^{++} and E.D.T.A.) are allowed to react with dinitrophenylhydrazine and NaOH. These standards are equivalent to 480 and 960 mμM oxoglutarate/ml. serum/hour at 37° C.

(If high values are expected the incubation time may be reduced to 15 min. A micro modification, with some loss in accuracy, is also possible using 0.1 ml. serum, the rest of the reagents in proportion, and ending with 2 ml. NaOH.)

The normal range with this method is 250–500 mμM oxoglutarate/ml. serum/hour at 37° C. The method appears to give results about four times as high as those obtained in TPNH units/ml. serum/hour at 25° C. by the Wolfson and Williams-Ashman spectrophotometric method.

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Serum Albumin, Pseudo Cholinesterase, and Transaminase in the Assessment of Liver Disease before and after Porta-caval and Lieno-renal Anastomosis

A. H. HUNT and H. LEHMANN (St. Bartholomew's Hospital, London) said that the analysis dealt with 155 cirrhotic patients who had undergone 157 shunt operations. The mild cirrhotics numbered 27, of whom 22 were completely successful and one considerably benefited, giving a total of 85% success. The operation was considered a failure if there was post-operative thrombosis or late liver degeneration. There were no post-operative deaths. The liver function has remained within normal limits during the whole period under which these cases have been under observation (up to 10 years).

The moderate cirrhotics numbered 88; 56 were successful, 22 had deteriorated over the years, but in 17 the porta-caval anastomosis benefited them, giving a total of 83% of cases materially improved by the anastomosis. Post-operative deaths were 10 (11%). In this group the serum albumin level pre-operatively was 4.4 g.%, and during the first four to five years after operation has not, on average, dropped below 4.1 g.%. The pseudo cholinesterase, 60 units (Warburg) pre-operative, has fluctuated during some four years between 58 and 64 units.

In those that deteriorated the serum albumin level pre-operatively was 4.3 g.%, and it did, on average, sink to 3.5 g.% before terminal deterioration. Of the 10 who died post-operatively, the pre-operative serum albumin level was 4.1 g.%, and death on the whole was not related to any deficiency in liver function but to technical difficulty.

In the advanced group, which numbered 40, the patients, when first seen, were not considered to be fit subjects for operation. Only after medical treatment for at least three months was porta-caval anastomosis deemed advisable; 20 (50%) did well. The serum albumin level during the pre-operative phase rose from an average of 2.5 g.% to an average of 3.8 g.%; and over the first three post-operative years it has fluctuated between 4 and 3.7 g.%. The pseudo cholinesterase, 46 units pre-operatively, has averaged between 52 and 54 units during the first three post-operative years, the conclusion being that if a patient responds well to medical treatment pre-operatively the prognosis is reasonably good, and that a prolonged course of medical treatment is imperative before deciding against operation. Eight advanced cirrhotics deteriorated post-operatively, but five were temporarily benefited, so that one can say that in 25 (or 62%) a shunt operation has been of great value. In those that deteriorated the serum albumin level has dropped from 3.8 g.% before to 3.4 g.% after operation, but the pseudo cholinesterase has risen on average from 37 to 45 to 51 units over the first two years. In the advanced cirrhotics who died post-operatively (12, constituting 30% of this group), the serum albumin averaged 3.5 g.% pre-operatively, and the pseudo cholinesterase 36 units.

The chief conclusions concerning the three laboratory tests are:

(1) That no patient should be submitted to shunt operation with a serum albumin level of less than 3.2 g.%.

(2) That the pseudo cholinesterase fluctuates more from case to case, but on the whole a level of 35 units is not good, though not prohibitive. Of all the liver function tests the serum albumin is the greatest help, and alterations in pseudo cholinesterase give a more immediate estimate of the patient's day-to-day or week-to-week response.

(3) The transaminase levels have varied to such an extent that we have not yet been able to assess their value. They have indicated activity of hepatic disease, but they do not indicate overall incapacity of liver function, which is what the surgeon wants to know.

Some Enzyme Activities in Normal and Neoplastic Colon

R. A. DALE (Hammersmith Hospital, London) described an attempt to determine the effects of neoplasia on the relative activities of enzymes concerned with respiration, glycolysis, and nucleic acid metabolism. The activity of one enzyme involved in each of these processes, namely, lactate dehydrogenase, aldolase, and DNase, respectively, was measured in normal mucosa and in carcinomata of the colon. The determinations were carried out on extracts of epithelial cells which were prepared relatively free from connective tissue, and are referred to both N and DNA as indices.

The ratios of the activities of lactate dehydrogenase, aldolase, and DNase in carcinomata to those in normal mucosa are, respectively, approximately 2.0, 0.8, and 1. The significance of these findings was discussed.

An Enzymic Test for the Diagnosis of Galactosaemia

J. H. WILKINSON (Westminster Medical School, London) said that the accumulation of galactose-1-phosphate in the red blood cells of galactosaemic patients, first noted by Schwarz, Golberg, Komrower, and Holzel (1956), led Kalckar and his co-workers to show that the basic defect in this disease is absence or deficiency of galactose-1-phosphate uridyl transferase. Subsequently Anderson, Kalckar, Kurahashi, and Isselbacher (1957) developed a spectrophotometric procedure for the determination of the activity of this enzyme in red cell haemolysates. This method was used during the present work.

The normal enzyme activity of healthy individuals was found to be in the range 5.9–12.8 units/g. haemoglobin. No activity could be detected in a haemolysate obtained from a patient with galactosaemia. This patient, now a healthy boy of 6, had his condition diagnosed in infancy and was reared on a galactose-free diet.

A second patient, a girl of 9 weeks, was found to excrete galactose in the urine and showed consider-

able clinical improvement when placed on a galactose-free diet. Certain other features of the case, however, did not entirely support the diagnosis of galactosaemia. In this case the transferase activity of the red cells was 6.8 units/g. haemoglobin, a value well within the normal range. It is suggested that in this case some other defect was responsible for the galactosuria after a milk feed.

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Schwarz, V., Golberg, L., Komrower, G. M., and Holzel, A. (1956). *Biochem. J.*, **62**, 34.

A New Urine Protein Abnormality

ELIZABETH A. BUTLER and F. V. FLYNN (Department of Clinical Pathology, University College Hospital, London). As a result of studying by starch gel electrophoresis the proteinuria that accompanies renal tubular disorders, a new urine protein fraction has been recognized. This migrates more slowly than the slowest γ globulin in normal serum.

The proteins of 206 urine specimens, concentrated by dialysis or ultrafiltration, were examined by electrophoresis on paper and starch gel, usually in parallel with the corresponding serum proteins. A few were also studied by immuno-electrophoresis. Specimens were obtained from normal controls and from 164 patients, including 31 with renal tubular dysfunction.

Among the 106 patients with clinical proteinuria, 36 had a small post-γ fraction on starch gel electrophoresis, including 14 with the Fanconi syndrome and nine with myelomatosis. In two a post-γ protein was detected in the serum also.

All those with post-γ protein in the urine were potentially liable to have some disturbance of bone metabolism, but there was no correlation with the results of calcium and alkaline phosphatase determinations. Nearly all had conditions that may be associated with renal tubular dysfunction, but evidence of resorption defects was lacking in some.

It was shown that the post-γ protein was unlikely to arise from denaturation, formation of a metal complex, genetic variation, genito-urinary tract secretions, or the breakdown of red cells, pus cells, or casts. In two cases of myelomatosis it was shown to be a Bence Jones protein, and since it apparently failed to react with an antiserum to normal serum proteins it may represent an abnormal protein in all.

Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. F. Hampson, Department of Pathology, Royal Berkshire Hospital, Reading,** price 1s. each.

No.	Title	Date	Author
1	The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics	1952	R. W. Fairbrother
2	Determination of Sensitivity of *M. tuberculosis* to Streptomycin	1952	R. L. Vollum
3	The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents	1953	L. C. Nickolls
4	The Estimation of Carbon Monoxide in Blood	1953	D. A. Stanley
5	The Identification of Reducing Substances in Urine by Partition Chromatography on Paper	1953	G. B. Manning
6	The Paul-Bunnell Test	1954	R. H. A. Swain
7	The Papanicolaou Technique for the Detection of Malignant Cells in Sputum	1955	F. Hampson
8	Investigation of Haemorrhagic States with Special Reference to Defects of Coagulation of the Blood	1955	E. K. Blackburn
9	Daily Fat Balance	1956	A. C. Frazer
10	Mycological Techniques: (1) Collection of Specimens	1956	R. W. Riddell
11	Mycological Techniques: (2) Cultural Isolation	1956	R. W. Riddell
12	Techniques for Demonstrating L.E. Cells	1956	J. V. Dacie and L. S. Sacker
13	The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis	1956	Joan Taylor
14	The Determination of Serum Iron and Serum Unsaturated Iron-binding Capacity	1956	Arthur Jordan
15	The Estimation of Faecal "Urobilinogen"	1957	C. H. Gray
16	Preservation of Pathological Museum Specimens	1957	L. W. Proger
17	Cultural Diagnosis of Whooping-cough	1957	B. W. Lacey
18	The Rose-Waaler Test	1957	C. L. Greenbury
19	The Laboratory Diagnosis of Fibrinogen Deficiency	1958	R. M. Hardisty
20	Investigation of Porphyrin	1958	C. Rimington
21	Quantitative Determination of Porphobilinogen and Porphyrins in Urine and Faeces	1958	C. Rimington
22	Investigation of Haemolytic Anaemia	1959	J. G. Selwyn
23	The Dried Disc Technique for Bacterial Sensitivity Tests	1959	R. W. Fairbrother and J. C. Sherris

BOOK REVIEWS

A Text on Systemic Pathology, Vol. II. Edited by Otto Saphir. (Pp. xvi+1,084; 979 figures. $38.) New York: Grune and Stratton. 1959.

This large, two-volume book is edited and written by a well-known team of pathologists. The method has been to divide the subject matter systemically and that has many benefits. Nevertheless, it has also the defects of its merits, particularly in overlapping between the sections. It would have been profitable if the editor had integrated the different sections and cut out as much as possible of the overlapping material when he had received all his contributions. Also, as so often happens when a book is written by a team of authors, the balance is occasionally somewhat odd; thus there are 32 pages devoted to hermaphroditism whereas the whole subject of infective hepatitis is dismissed in 12 pages. The whole effect is a series of authoritative single articles on specialist themes which are most useful for the specialist but retain this air of inadequacy as a reference book for a large field of study.

The book is most excellently produced, particularly the photomicrographs. A. GORDON SIGNY.

A Ciba Foundation Symposium on **Amino Acids and Peptides with Antimetabolic Activity.** Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii+286; 28 figures. 45s.). London: Churchill. 1958.

This symposium in March, 1958, brought together workers in the two fields of tumour inhibitors and of antibiotics under the chairmanship of Professor Bergel. There were 30 participants from nine countries, and 17 papers were given. The discussions ranged from the chemistry and synthesis of these agents through their mode of action to their therapeutic effect. This is a highly specialized book, and although it will be of great interest to workers in these fields it is heavy going for the general reader. G. K. MCGOWAN.

Clinical Flame Photometry. By Henry A. Teloh. (Pp. xvi+103; 36 tables, 9 figures. 34s.). Illinois: Charles C. Thomas; Oxford: Blackwell Scientific Publications. 1959.

The estimation of the blood electrolytes is an everyday procedure largely due to the introduction of a rapid method for the estimation of sodium and potassium by making use of the flame photometer. Dr. H. A. Teloh, of the Northwestern University Medical School, Chicago, has written a small manual describing some of the methods available for sodium, potassium, calcium, and magnesium. There are a few chapters on the theory of emission spectrophotometry, the general design of the instruments, and some of the dangers inherent in the techniques.

Provided one is fortunate in possessing a Beckman apparatus the methods are valuable, but only two methods use other models, one a German and the other an English, but some of the techniques could be adapted for equipment available outside America. However, the various methods recommended are well described and could be readily followed.

It seemed to the reviewer unfortunate that Figs. 5 and 6 should be presented some 20 pages after being mentioned, and also after the appearance of Figs. 7 and 8. Another small feature which to the English reader is unusual is the use of gm. instead of g. as the abbreviation for a measure of weight.

Despite these minor criticisms the book can fill an important place on the shelves of the hospital biochemist, and will be of much practical value to him. J. N. CUMINGS.

BOOKS RECEIVED

Biomicroscopie du Corps Vitré et du Fond de l'Oeil. By A Busacca, H. Goldmann, and S. Schiff-Wertheimer. (Pp. 382; 144 figures, 40 colour plates. Fr. fr. 8,000.) Paris: Masson. 1957.

An Introduction to Medical Laboratory Technology, 2nd ed. By F. J. Baker, R. E. Silverton, and Eveline D. Luckcock. (Pp. xiv+408; 118 figures. 35s.) London: Butterworth. 1957.

The Planning and Organization of Central Syringe Services. (Pp. 59. 2s. 6d.) London: The Nuffield Provincial Hospitals Trust. 1958.

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